Studies on the microbial halogen cycle:

Reactions of fungal peroxidases and bacterial reductive dehalogenases

Dissertation

to obtain the academic degree of
doctor rerum naturalium
(Dr. rer. nat.)

at
the Faculty of Biology and Pharmacy
of the Friedrich-Schiller- University Jena

submitted

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Date of the public defence: 12.03.2010

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Abbreviations

ABTS	2,2'-**A**zino-**B**is(3-ethylbenz**T**hiazoline-6-**S**ulphonic acid)
AOX	**A**dsorbable **O**rganic Halogen
APS	**A**mmonium **P**er**S**ulphate
2-BP	2-**B**romo**P**henol
4-BP	4-**B**romo**P**henol
BrPO	**Br**ominating **P**er**O**xidase
*c*DBE	***c****is*-1,2-**D**i**B**romo**E**thene
*c*DCE	***c****is*-**D**i**C**hloro**E**thene
CHBA	3-**C**l-4-**H**ydroxy**B**enzo**A**te
ClOHPA	3-**Cl**-4-**OH**-**P**henyl**A**cetate (=3-Cl-4-hydroxy-phenylacetate)
2-CP	2-**C**hloro**P**henol
DBE	**D**i**B**romo**E**thene
2,3-DBP	2,3-**D**i**B**romo**P**ropene
2,6-DCA	2,6-**D**i**C**hloro**A**nisole
DCHBA	3,5-**D**i**C**hloro-4-**H**ydroxy**B**enzo**A**te
2,3-DCP	2,3-**D**i**C**hloro**P**ropene
2,6-DCP	2,6-**D**i**C**hloro**P**henol
DMP	2,6-**D**i**M**ethoxy**P**henol
dNTP	**d**eoxyribo**N**ucleotide **T**ri**P**hosphate
DTT	1,4-**D**i**T**hio-D,L-**T**hreitol
EDTA	**E**thylene**D**iamine**T**etra**A**cetic acid
GC	**G**as **C**hromatography
HEPES	N-(2-**H**ydroxy**E**thyl)**P**iperazine-N'-2-**E**than**S**ulfonic acid
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
HPO	**H**alo**P**er**O**xidase
LC	**L**iquid **C**hromatography
LiP	**Li**gnin **P**eroxidase
MALDI	**M**atrix-**A**ssisted **L**aser **D**esorption/**I**onization
MCD	**M**ono**C**hloro**D**imedone
MnP	**M**anganese **P**eroxidase
MS	**M**ass **S**pectrometry
MV	**M**ethyl **V**iologen
NBT	*p*-**N**itro**T**etrazolium **B**lue chloride
NMR	**N**uclear **M**agnetic **R**esonance
OD	**O**ptical **D**ensity
OHPA	4-**OH**-**P**henyl**A**cetate (=4-hyroxy-phenylacetate)
PAGE	**P**oly**A**crylamid **G**el **E**lectrophoresis
PBST	**P**hosphate **B**uffered **S**aline + **T**ween 20
PCE	**P**er**C**hloro**E**thene (=tetrachloroethene)
PceA	PCE reductive dehalogenase
$PceA^-$	PCE reductive dehalogenase negative
PCR	**P**olymerase **C**hain **R**eaction

PVDF membrane	**P**oly**V**inyli**D**ene **F**luoride membrane
SDS	**S**odium **D**odecyl **S**ulfate
TBE	**T**ri**B**romo**E**thene
TCE	**T**ri**C**hloro**E**thene
*t*DBE	***t****rans*-1,2-**D**i**B**romo**E**thene
TEMED	N,N,N',N'-**TE**tram**M**ethyl**E**thylen**D**iamine
TFA	**T**ri**F**luoro**A**cetic acid
TMDPQ	3,3',5,5'**-T**etra**M**ethoxy-4,4'-**D**i**P**heno**Q**uinone
TOF	**T**ime-**O**f-**F**light mass spectrometer
TOX	**T**otal **O**rganic **H**alogen
Tris-HCl	**Tris**(hydroxymethyl)aminomethane Hydrochloride (=**HCl**)
VA	**V**eratr**A**ldehyde
VB	**V**inyl **B**romide
VP	**V**ersatile **P**eroxidase
X-phosphate	5-bromo-4-chloro-3-indolylphosphate-p-toluidin salt

Summary

Halogenated metabolites are produced e. g. by lignin degrading fungi and may be available to organohalide respiring anaerobes in natural environments. However, it is unclear if there is any connection between aerobic biological halogenation and anaerobic dehalogenation. The aims of the present study were to answer this question and to shed some light on microbial halogenation and dehalogenation involving halide ions other than the well studied chloride (e. g. bromide, fluoride).

Using *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* strain PCE-S and their purified tetrachloroethene (PCE) reductive dehalogenases as a model system, dehalogenation of brominated alkenes was investigated and compared with that of their chlorinated analogues. In previous studies it was shown that PCE dehalogenation mediated by these organisms occurred at high stereospecificity and led to the formation of *cis*-1,2-dichloroethene (*c*DCE) as the end product. In this study, an isomer mixture of the brominated analogue of *c*DCE, *cis*-dibromoethene (*c*DBE) and *trans*-DBE, was found to serve as electron acceptors for both organisms and even support growth of *S. multivorans* in the presence of formate as electron donor. The DBE isomers are reported here for the first time to be respiration substrates for *S. multivorans* as well as inductors for the PCE dehalogenase synthesis. The purified PCE dehalogenases debrominated both isomers of DBE to vinyl bromide (VB) with *t*DBE being dehalogenated prior to *c*DBE. With *D. hafniense* PCE-S and its PCE dehalogenase, ethene was also formed. Tribromoethene was debrominated by the PCE dehalogenases via all three isomers of DBE (1,2-*cis*-, 1,2-*trans*-, and 1,1-DBE) as intermediates to VB (as well as ethene in case of *D. hafniense* PCE-S). The finding that different DBE isomers were formed as debromination products indicated a lower stereospecificity of the debromination as compared with the dechlorination.

Studies on the dehalogenation of halogenated alkenes indicated that the reaction mechanism of dechlorination and debromination appeared to be very similar. Independent on the type of halogen substituent, dehalogenation of the halogenated propenes probably led to the formation of methyl viologen adducts with putative halopropenyl radical intermediates, whereas no such adducts appeared to form in dehalogenation of the halogenated ethenes. In addition, none of the halogenated propenes tested were able to support growth or to induce the enzyme synthesis; also, ammonia did

not stimulate dehalogenation of the halogenated propenes, whereas the presence of ammonia significantly stimulated dehalogenation of the halogenated ethenes. These results indicated that the carbon backbone has a more significant effect on the reaction mechanism and on the induction of the enzyme synthesis than the type of halogen.

As a model for halogenating fungi, *Bjerkandera adusta* strain Ud1 was studied for halogenating enzyme production. Up to now, no halogenating enzyme has been found in *B. adusta*, despite the fact that the synthesis of halogenated metabolites by this fungal species has been studied for decades. The versatile peroxidases (VPs) purified in this study exhibited bromoperoxidase activity with monochlorodimedone, phenol red and phenol as substrates. The brominating activity of VPs indicates that halogenation might be a side reaction of ligninolytic peroxidases. Since organohalides have been reported to be good substrates for H_2O_2-producing enzymes, this observation might point to an important role of halogenation in lignin degradation.

For the first time, a fluoride-dependent activity was observed with the versatile peroxidases as well as manganese peroxidases (MnPs) from *B. adusta*. This fluoride-dependent activity was also found with the MnPs of three other phylogenetically different fungal species. Using MnP-1 from *B. adusta* strain Ud1, a variety of substrates were found to be fluoride-dependently converted in the absence of Mn(II). The velocity of these reactions increased with increasing concentration of fluoride; high concentrations of fluoride (20 mM) were required to obtain the same effect as with low concentrations of Mn(II) (0.15 mM). Neither hypofluorus acid nor fluorinated reaction products were formed. The substrates, e. g. monochlorodimedone and 2,6-dimethoxyphenol, were oxidized as indicated by GC-MS and LC-MS analyses. The same reaction products, a dimer and an *O*-demethylated dimer, were formed from 2,6-dimethoxyphenol by MnP-1 in the presence of either F^- or Mn(II). Fluoride was found to bind to MnP in competition with hydrogen peroxide. Similar pH dependence was observed for fluoride binding and the fluoride-dependent reactions. The presence of Mn(II) significantly inhibited the fluoride-dependent reactions. From these results it was concluded that fluoride binding might have triggered a conformational change in a few amino acids close to the fluoride binding site, which led to the activation of a long-range electron transfer pathway, as previously described for versatile peroxidases and lignin peroxidases. This enabled the MnP to oxidize the substrates on the periphery of the enzyme, which had no binding sites near the heme pocket.

Halogenated metabolites were produced by *B. adusta* strain Ud1, identified as 3-Cl-*p*-anisaldehyde and 3,5-dichloro-4-methoxybenzaldehyde by GC-MS. To test a potential connection between fungal halogenation and bacterial reductive dehalogenation, 3-Cl-*p*-anisaldehyde and 3,5-dichloro-4-methoxybenzoate (the oxidized form of 3,5-dichloro-4-methoxybenzaldehyde) were fed to *D. hafniense* strain DCB-2. The bacteria could *O*-demethylate both fungal metabolites. Dehalogenation was also observed when 3,5-dichloro-4-methoxybenzoate was supplied as substrate. Furthermore, an anaerobic mixed culture with *O*-demethylating and dehalogenating activity was enriched from forest soil. A pure culture isolated from this enrichment culture was identified as *D. hafniense*. In the same soil sample, ligninolytic enzyme activities were detected. These experiments point to an interaction between aerobic halogenating fungi and anaerobic dehalogenating bacteria.

Zusammenfassung

Halogenierte Metabolite werden z.B. von ligninabbauenden Pilzen gebildet. Sie könnten für anaerobe Bakterien, die eine Organohalidatmung durchführen im natürlichen Habitat verfügbar sein. Es ist jedoch unklar, ob es tatsächlich eine Verbindung zwischen aerober biologischer Halogenierung und anaerober Dehalogenierung gibt. Ziel der hier vorgelegten Untersuchungen ist es, eine Antwort auf diese Frage zu geben sowie die Rolle der in die mikrobielle Halogenierung und Dehalogenierung involvierten Halidionen (z.B. Bromid, Fluorid) ergänzend zu den gut untersuchten Chloriden zu beleuchten.

Sulfurospirillum multivorans und *Desulfitobacterium hafniense* strain PCE-S und ihre gereinigten reduktiven Tetrachloroethen (PCE) Dehalogenasen wurde als Modellsysteme verwendet. Die Dehalogenierung bromierter Alkene wurde untersucht und mit der ihrer chlorierten Analoga verglichen. In früheren Studien konnte gezeigt werden, dass die von diesen Organismen ausgeführte PCE Dehalogenierung mit hoher Stereospezifität erfolgt und zur Bildung von *cis*-1,2.dichloroethen (*c*DCE) als Endprodukt führt. Eine Isomerenmischung bromierter Analoga des *c*DCE, *cis*-Dibromoethene (*c*DBE) und *trans*-DBE kann als Elektronenakzeptor für beide Organismen dienen und in Anwesenheit von Formiat als Elektronendonor auch das Wachstum von *S. multivorans* unterstützen. Es wurde erstmals gezeigt dass die DBE Isomere sowohl Atmungssubstrate für *S. Multivorans* als auch als Induktoren der Synthese der PCE Dehalogenase sind. Die gereinigte PCE Dehalogenasen debromierten beide Isomere des DBE zum Vinylbromid (VB). *t*DBE wurde dabei vor *c*DBE debromiert. Es konnte festgestellt werde, dass *D. hafniense* PCE-S und seine PCE Dehalogenase auch Ethen bilden. Tribromoethen wurde durch die PCE Dehalogenasen über die drei möglichen isomeren Intermediate des DBE (1,2-*cis*-, 1,2-*trans*- und 1,1-DBE) zum Vinylbromid und im Falle von *D. hafniense* PCE-S auch zum Ethen debromiert. Die Feststellung, dass verschiedene DBE Isomere als Debromierungsprodukte gebildet werden ist ein Hinweis auf die im Vergleich zur Dechlorierung geringere Stereospezifität der Debromierung.

Untersuchungen zur Dehalogenierung halogenierter Ethene zeigen, dass der Rektionsmechanismus von Dechlorierung und Debromierung sehr ähnlich zu sein scheint. Unabhängig vom Typ des Halogensubstituenten führt die Dehalogenierung halogenierter Propene wahrscheinlich

zur Bildung von Methylviologenaddukten mit Halopropenylradikalen als möglichen Intermediaten, während solche Addukte bei der Dehalogenierung halogenierter Ethene anscheinend nicht gebildet werden. Darüberhinaus war keines der getesteten halogenierten Propene in der Lage das Wachstum zu unterstützen oder die Enzymsynthese zu induzieren; auch Ammonium stimulierte die Dehalogenierung halogenierter Propene im Unterschied zu halogenierten Ethenen nicht. Diese Ergebnisse zeigen, dass das Kohlenstoffskelett einen größeren Einfluss auf den Reaktionsmechanismus und die Induktion der Enzymsynthese hat als der Typ des Halogensubstituenten.

Als Modell für halogenierende Pilze wurde *Bjerkandera adusta* Ud1 hinsichtlich der Bildung halogenierender Enzymaktivitäten untersucht. Bis heute wurden in *B. adusta* keine halogenierenden Enzyme nachgewiesen obwohl die Synthese halogenierter Metabolite durch diesen Pilz bereits intensiv untersucht worden ist. Mit Monochlorodimedone, Phenolrot und Phenol als Substrat wurde gezeigt, dass die in dieser Untersuchung gereinigten Versatilen Peroxidasen (VPs) Bromierungsaktivität besitzen. Die Bromierungsaktivität Versatiler Peroxidase zeigt, dass Halogenierungen Nebenreaktionen ligninolytischer Peroxidasen sein können. Da Organohalide als gute Substrate für H_2O_2-produzierende Enzyme gelten, könnte diese Beobachtung ein Hinweis auf die bedeutende Rolle der Halogenierung für den Ligninabbau sein.

Erstmals wurden sowohl in Versatilen Peroxidasen als auch in Manganperoxidasen (MnPs) aus *B. adusta* fluorid-abhängige Aktivitäten beobachtet. Diese fluorid-abhängigen Aktivitäten wurden auch bei MnPs aus drei anderen phylogenetisch verschiedenen Pilzspezies gefunden. Es konnte gezeigt werden, dass MnP-1 von *B. adusta* Stamm Ud1 eine Reihe von Substraten fluoridabhängig in Abwesenheit von Mn(II) umsetzt. Die Geschwindigkeit dieser Reaktion steigt mit steigender Fluoridkonzentration; hohe Fluoridkonzentrationen (20 mM) sind erforderlich um den gleichen Effekt wie in Anwesenheit niedriger Mn(II) Konzentrationen (0,15 mM) zu erreichen. Es wurden weder hypofluorige Säure noch fluorierte Reaktionsprodukte gebildet. Mittels GC-MS und LC-MS konnte gezeigt werden, dass die Substrate Monochlorodimedon und 2,6-dimethoxyphenol oxidiert werden. In Anwesenheit von H_2O_2 und entweder Mn(II) oder F^- wird von MnP-1 aus 2,6-dimethoxyphenol jeweils das gleiche Reaktionsprodukt gebildet. Es wurde gefunden, dass Fluorid kompetitiv zu H_2O_2 am Enzym bindet. Fluoridbindung und fluoridabhängige Reaktionen zeigten die gleiche pH-Abhängigkeit. In Anwesenheit von Mn(II) ist die fluorid-abhängige Reaktion signifikant gehemmt. Aus diesen Ergebnissen ergibt sich die Schlussfolgerung, dass die Bindung

von Fluorid Konformationsänderungen einiger Aminosäuren nahe der Fluoridbindestelle verursachen, die letztlich zur Aktivierung eine long-range Elektronentransferweges , wie er u.a. für Versatile Peroxidasen und Ligninperoxidasen beschrieben ist, führt. In der Folge ist dann eine Oxidation von Substraten, für die MnP sonst keine Bindungsstelle besitzt, an der Peripherie des Enzyms möglich.

Von *B. adusta* Stamm Ud1 produzierte halogenierte Metabolite wurden mittels GC-MS als 3-Cl-p-anisaldehyd und 3,5-dichloro-p-anisaldehyd identifiziert. Um eine potentielle Verbindung zwischen pilzlicher Halogenierung und bakerieller reduktiver Dehalogenierung zu prüfen, wurden 3-Cl-p-anisaldehyd und 3,5-dichloro-4-methoxybenzoesäure (Oxidationsprodukt des 3,5-dichloro-p-anisaldehyd) als Substrat für *D. hafniense* Stamm DCB-2 verwendet. Die Bakterien können diese pilzlichen Metabolite *O*-demethylieren. Für die Bakterienkultur mit dem Substrat 3,5-dichloro-4-methoxybezoesäure wurde auch eine Dehalogenierung beobachtet. Weiterhin wurde eine anaerobe Mischkultur mit *O*-demethylierender und dehalogenierender Aktivität aus Waldboden angereichert. Aus dieser Anreichungskultur wurde eine Reinkultur isoliert und als *D. Hafniense* identifiziert. In der Bodenprobe wurden gleichzeitig auch ligninolytische Enzymaktivitäten nachgewiesen. Diese Untersuchungen weisen auf die Existenz einer Verbindung zwischen aerob halogenierenden Pilzen und anaerob dehalogenierenden Bakterien hin.

1. Introduction

1.1 Production and decomposition of natural organohalogens

The halogen cycle is now being recognized as one of the major complex biogeochemical cycles, since in the last few decades it has been shown that a huge number and variety of halogenated organic compounds of anthropogenic, biogenic or geogenic sources have been released into the environment (Öberg, 2002). Until the early 1970s, it was believed that the formation of carbon-halogen bonds in living organisms happened only rarely. However, over the last few decades, an increasing number of natural organohalogen compounds has been described, from about 200 in 1970s to more than 3700 in 2003 (Gribble, 2003) and approximately 4000 in 2009 (Wagner *et al.*, 2009), with structural classes ranging from simple phenolic and aliphatic compounds to complex polyketides and oligopeptides.

Chlorinated and brominated compounds are the two major groups of natural occurring organohalogens. Marine organisms, mainly algae and invertebrates produce bromine-containing metabolites as the major organohalogens due to the relatively high bromine concentration in marine environments compared to terrestrial systems (van Pée, 1996), whereas soil bacteria (Ehrlich *et al.*, 1947), fungi (de Jong *et al.*, 1992) and some higher plants (Engvild *et al.*, 1981) preferentially produce chlorine-containing metabolites.

Iodinated compounds occur less frequently, although they are also produced by many different organisms, e. g. mammals (Harington and Barger, 1986) and marine algae (Siuda and DeBernardis, 1973).

Fluorinated metabolites are very rare. The fluoride anion - compared to chloride or bromide - is a poor nucleophile in aqueous solution. Hence, it is not surprising that incorporation of fluorine in organic compounds by haloperoxidases has not been observed so far (Murphy, 2003) and only few fluorinated natural compounds have been found in bacteria (Sanada *et al.*, 1986) and a few higher plants (Oelrichs and McEwan, 1961).

In accordance with the very high number of naturally occurring organohalogens, the number and diversity of organohalogen producing organisms are also very high. Halogenated compounds are produced by microorganisms, algae, plants, marine invertebrates, insects and mammals (in-

cluding humans) (for a review, see van Pée, 2001).

The synthesis of natural organohalogens is part of the global halogen cycle. Besides the natural production of organohalogens of biological or geological source, halogenated compounds are produced by the chemical industry. Anthropogenic organohalogens used e. g. as biocides in agriculture or solvents and cleaning agents in industry are frequently found as pollutants of natural environments. Because of acute and chronic toxicity, persistence, and bioaccumulation, the presence of organohalides in the environment is of major concern and becomes a threat to human and environmental health (Ahlborg and Thunberg, 1980; Hileman, 1993; Henschler, 1994). There is a demand for processes that can dehalogenate and thus detoxify organohalides in these environments.

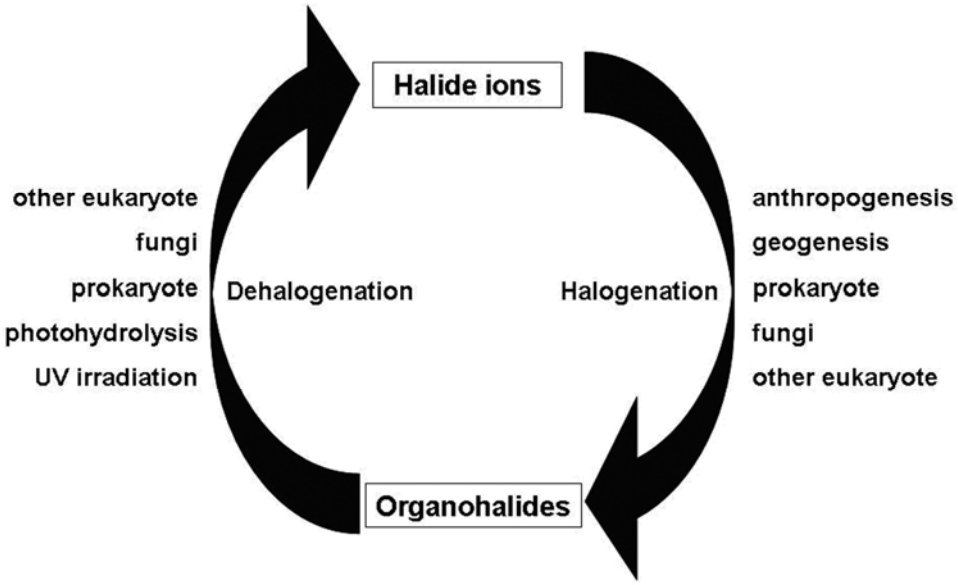

Fig. 1.1 Simplified scheme of halogen cycle.

Since carbon-halogen bonds can be synthesized naturally, they should also be broken naturally (Fig. 1.1). Organohalogens could be decomposed in both abiotic and biotic processes such as ultraviolet irradiation in the atmosphere, photohydrolysis near the aquatic surface, and biological degradation by prokaryotes, fungi, plants, nematodes, mammals and other eukaryotic organisms (Castro, 1998; Häggblom and Bossert, 2001). Quantitatively, the most important transformation of organohalogens in terrestrial and aquatic environments is the dehalogenation mediated by microorganisms, primarily bacteria (Castro, 2003). Halogenated aliphatic and aromatic compounds may be degraded by microbes in metabolic or cometabolic or even abiotic processes, the latter being

mediated by cofactors such as corrinoids or hemes (for reviews, see Wohlfahrt and Diekert, 1997; Smidt and de Vos, 2004). Organohalides may also serve as carbon and energy sources for specialized dehalogenating microorganisms, thus representing a type of feedstocks in microbial food webs (Löffler *et al.*, 2003).

Probably organohalogen compounds of natural rather than recent anthropogenic origin have primed the development of a broad repertoire of microbial systems to dehalogenate or completely mineralize those molecules to prevent their accumulation in nature. Recent biogeographic studies have reinforced this possibility by revealing the global distribution of dehalogenating populations and their occurrence in polluted as well as pristine environments (Lanthier *et al.*, 2001; Hendrickson *et al.*, 2002).

1.2 Microbial halogenation

The first halogenated metabolite of microbial origin, diploicin, was described in 1904 (Zopf *et al.*, 1904). Microbial halogenation has been recognized as a major source of naturally occurring organohalides.

1.2.1 Bacterial halogenation

Most halogenated bacterial metabolites are connected with antibiotic activities. The structural variety of bacterial halogenated metabolites is very broad, with aromatic compounds as the large majority. Soil bacteria usually synthesize chlorine-containing metabolites while bacteria in marine system tend to produce brominated compounds (for a review, see van Pée, 1996). Only very few fluorinated (Oelrichs and McEwan, 1961; Sanada *et al.*, 1986) or iodinated compounds (see e. g. Lee *et al.*, 1989) have been reported to be produced by bacteria.

Since the search for organohalogens produced by bacteria is always connected with biological activities, especially antibiotic activity, most halogenated metabolites described so far are produced by the antibiotic-producing actinomycetes, including species from *Streptomyces, Actinoplanes* and *Nocardia*. Besides, a few halogenated metabolites are synthesized by pseudomonads (van Pée, 1996) and members of other genera, e. g. myxobacteria (Gerth *et al.*, 1982). These organohalogens are usually products of the secondary metabolism. Aside from the function as antibiotics,

other physiological functions of these compounds are still not very clear.

1.2.2 Fungal halogenation

In terrestrial environments, white rot fungi, especially basidiomycetes, are known for their ubiquitous capacity to produce organohalogen metabolites (Field and Wijinberg, 2003). Many basidiomycetes can produce large amounts of low-molecular-weight organohalogens or the so-called adsorbable organic halogens (AOX) when grown on lignocelluloses substrates or synthetic media. A broad taxonomic distribution was found across 68 genera from 20 families within the orders of the Agaricales and Aphyllophorales (Table 1.1). A high biodiversity of the biohalogenating capacity has been suggested by de Jong and Field (1997), considering the broad capacity of basidiomycetes to produce organohalogens in combination with the absence of correlation in organohalogen production between closely related species.

Basidiomycetes are an ecologically significant group, which accounts for a large proportion of the living biomass in plant litter (Swift, 1982). Therefore, halogenated compounds produced by this class of organisms might play an important role in the global halogen cycle. As potent decomposers of forest litter, basidiomycetes are actually suggested as a major source of natural organohalogens in terrestrial environments (de Jong and Field, 1997). Many of the organohalogen metabolites of basidiomycetes are produced in high amounts while growing on natural substrates and are therefore found in natural environments such as forest litter (de Jong *et al.*, 1994a; Verhagen *et al.*, 1998a).

Most of the halogenated metabolites identified from basidiomycetes to date are chlorinated, although brominated and iodated metabolites have also been described. These organohalide metabolites could be divided into two broad categories: the halogenated aromatic compounds and the haloaliphatic compounds. The chlorinated aromatic compounds are the major group, including chlorinated phenyl methyl ethers (de Jong and Field, 1997).

During the last few decades, new roles for organohalogens produced by basidiomycetes have been described, varying from antibiotics (Kavanagh *et al.*, 1952) to substrates for hydrogen peroxide production by aryl alcohol oxidases (de Jong *et al.*, 1994b) and educts for biosynthesis of secondary metabolites (Harper, 2000).

Table.1.1 Low-molecular-weight organohalogens and/or AOX[1] and TOX[2] produced by basidiomycetes (modified from de Jong and Field, 1997)

Family	Agaricales Genus	Halogenated metabolites	Family	Aphyllophorales Genus	Halogenated metabolites
Agaricaceae	*Agaricus*	AOX, chloromethane, drosophilin A, drosophilin A methyl ether	Cantharellaceae	*Cantharellus*	chloroform
			Clavariaceae	*Macrotyphula*	AOX
Amanitaceae	*Amanita*	AOX, chlorinated nonprotein amino acids	Coniophoraceae	*Coniophora*	AOX
Bolbitiaceae	*Agrocybe*	AOX		*Serpula*	AOX
Boletaceae	*Boletus*	AOX, chlorocresol	Corticiaceae	*Phlebia*	TOX
	Xerocomus	AOX		*Meripilus*	AOX
Coprinaceae	*Coprinus*	AOX, drosophilin A		*Peniophora*	chlorinated anisyl aldehydes, drosophilin A, drosophilin A methyl ether, AOX
	Panaeolus	AOX			
	Psathyrella	AOX, drosophilin A			
Cortinariaceae	*Cortinarius*	5-Chlorodermorubin		*Resinicium*	pinicoloform
	Dermocybe	5-chlorodermolutein, 5-Chlorodermorubin	Ganodermataceae	*Ganoderma*	TOX
	Galerina	AOX	Gomphaceae	*Ramaria*	chlorinated anisyl aldehydes
	Gymnopilus	AOX	Hericiaceae	*Hericium*	AOX, Chlorinated orcinol methyl ethers
	Hebeloma	AOX			
Lepiotaceae	*Lepiota*	lepiochlorin	Hymenochaetaceae	*Fomitopora*	chloromethane
	Leucoagaricus	chlorinated isobenzofuranone		*Hymenochaete*	chloromethane
Russulaceae	*Lactarius*	1-chloro-5-heptadecyne		*Inonotus*	chloromethane
	Russula	AOX, chlorinated hydroquinone methyl ethers		*Onnia*	chloromethane
Strophariaceae	*Hypholoma*	AOX, chlorinated anisyl metabolites, drosophilin A methyl ether		*Phellinus*	AOX, TOX, chloromethane, bromomethane, iodomethane, 3,5-dichloro-4-methoxybenzyl alcohol, chlorinated hydroquinone methyl ethers, 1,2,3-trimethoxy-4,5,6-trichlorobenzene
	Kuehneromyces	AOX, methyl 3,6-dichloro-2-methylbenzoate			
	Pholiota	AOX, chlorinated anisyl metabolites			
	Psilocybe	AOX			

	Stropharia	AOX, chlorinated anisyl metabolites		*Phylloporia*	AOX, 3,5-dichloro-4-methoxybenzaldehyde
Tricholomataceae	*Armillaria*	AOX, TOX, Chlorinated orsellinate sesquiterpenes	Polyporaceae	*Bjerkandera*	AOX, TOX, chlorinated anisyl metabolites, chlorinated hydroquinone methyl ethers, 4-Cl-3,5-dimethoxybenzaldehyde, 2,4-dichlorobenzoate
	Baeospora	AOX			
	Calocybe	AOX			
	Clitocybe	AOX, Chlorinated orsellinate sesquiterpenes			
	Collybia	AOX		*Daedaleopsis*	3-Cl-4-anisaldehyde
	Flammulina	AOX		*Fomes*	AOX, 3-Cl-*p*-anisaldehyde
	Laccaria	AOX		*Fomitopsis*	AOX, chloromethane
	Lentinellus	AOX, 2-Cl-3-(4-methoxyphenyl)-2-propen-1-ol		*Gloeophyllum*	AOX
	Lepista	AOX, TOX, chlorinated anisyl metabolites, chlorinated hydroquinone methyl ethers, miscellaneous chlorinated aromatic compounds		*Heterobasidion*	AOX, chlorinated benzenoid metabolite
				Ischnoderma	AOX, 3-Cl-*p*-anisaldehyde
	Macrocystidia	AOX		*Phaeolus*	chloromethane
	Marasmius	AOX, chlorinated anisyl metabolite		*Poria*	AOX, TOX, methyl chloro-4-methoxycinnamate
	Mycena	AOX, chlorinated anisyl metabolites, drosophilin A methyl ether, chlorinated strobilurin B, mycenon		*Schizopora*	AOX
	Oudemansiella	AOX, chlorinated anisyl metabolite		*Trametes*	AOX, TOX, 3-Cl-*p*-anisaldehyde,
	Pleurotus	AOX, 3-Cl-*p*-anisaldehyde			
	Strobilurus	chlorinated strobilurin B	Schizophyllaceae	*Schizophyllum*	drosophilin A
	Tricholomopsis	AOX			
	Xerula	AOX, chlorinated strobilurin B			

[1]AOX, absorbable organic halogen; [2]TOX, total organic halogen.

1.2.3 Halogenating enzymes

Halogenating enzymes have been found in a broad range of organisms, e. g. bacteria and fungi. They may be grouped into two major classes: (i) highly substrate-specific halogenases requiring dioxygen for enzymatic activity, using either flavin ($FADH_2$-dependent halogenases) or α-ketoglutarate (non-heme Fe(II)/α-ketoglutarate/ O_2-dependent halogenases) to function as co-substrates; and (ii) less specific haloperoxidases (HPO) utilizing hydrogen peroxide to form hypohalous acid together with halide ions (Wagner *et al.*, 2009).

As summarized in Table 1.2, the only enzyme capable to fluorinate discovered so far is a fluorinase isolated from *Streptomyces cattleya*, which catalyzes the fluorination of S-adenosylmethionine to form 5'-fluoro-5'-deoxyadenosine (Dong *et al.*, 2004). The fluorination catalyzed by this enzyme involves a mechanism which is absolutely different from that of the halogenation reactions mediated by haloperoxidases (chlorination, bromination or iodination). Fluorination takes place via nucleophilic attack of fluoride to the substrate; this reaction does not involve hydrogen peroxide, whereas halogenation with other halides is mostly mediated via hypohalous acid or halide radicals formed from the corresponding halide ion in the presence of hydrogen peroxide or dioxygen (Table 1.2, see also Dunford *et al.*, 1987).

Table 1.2 Halogenating enzymes. αKG, α-Ketoglutarate.

Enzyme	Requirements	Mechanism	Hal^-	Sources	Reference
Haloperoxidases	substrates with a double bond, H_2O_2, halide ions; heme or vanadate as cofactor	via hypohalous acid as halogenating agent	Cl^-, Br^-, I^-	fungi; bacteria; polychaete; algae	Hager *et al.*, 1966 ; Wiesner *et al.*, 1988 ; Chen *et al.*, 1991 ; Almeida *et al.*, 2000
Flavin-dependent halogenases	substrates with a double bond, O_2, halide ions; $FADH_2$	via hypohalous acid as halogenating agent	Cl^-, Br^-	*Pseudomonas* spp.; *Streptomyces* spp.	Keller *et al.*, 2000; Zehner *et al.*, 2005
α-Ketoglutarate-dependent halogenases	aliphatic substrates, αKG, oxygen, halide ions	via a chloride radical	Cl^-	*Pseudomonas* spp.	Vaillancourt *et al.*, 2005
Fluorinase	*S*-adenosylmethio-nine, halide ions	nucleophilic attack of halide	F^-, Cl^-	*Streptomyces cattleya*	Dong *et al.*, 2004

The first halogenating enzyme, the heme-containing chloroperoxidase of the fungus *Caldariomyces fumago*, was described by Shaw and Hager (1961). Using the photometric haloperoxidase assay developed by these scientists, many other chloro-, bromo- or iodoperoxidases have subsequently been detected and isolated from bacteria, fungi, marine algae, marine invertebrates and mammals (for a review, see van Pée and Unversucht, 2003). However, the role of haloperoxidases in many organisms is still poorly understood. Although there is some evidence supporting the involvement of haloperoxidases in the production of some natural organohalogens (Beissner *et al.*, 1981; Nunez, 1984), haloperoxidase-catalyzed halogenations lack substrate specificity and regioselectivity, which is inconsistent with the apparent specific halogenations required for the biosynthesis of many halogenated metabolites. Hence, the physiological and ecological functions of these widespread halogenating enzymes need further investigation.

Flavin-dependent halogenases and α-Ketoglutarate-dependent halogenases are regarded to be substrate-specific. Evidence has been provided for their involvement in the synthesis of several organohalogens (for a review, see Wagner *et al.*, 2009). All of these enzymes are isolated from antibiotics-producing bacteria. However, such substrate-specific halogenases can only be detected if their natural substrates are known, which hampers the discovery of novel enzymes.

1.3 Biological dehalogenation

1.3.1 Categories of biological dehalogenation

During the last few decades, dehalogenation reactions mediated by microorganisms have been studied in detail. There are several categories of biological dehalogenation with respect to their catalytic mechanisms: hydrolytic dehalogenation, thiolytic dehalogenation, intramolecular substitution, dehydrohalogenation, dehalogenation by hydration, dehalogenation by methyl transfer, oxidative dehalogenation and reductive dehalogenation (van Pée and Unversucht, 2003). The major types are listed in Table 1.3.

Polychlorinated organohalides such as polychlorinated biphenyls and tetrachloroethene are electron-deficient compounds and are thus more susceptible to reductive rather than oxidative attack (Smidt and de Vos, 2004). Therefore, reductively dehalogenating anaerobes are believed to play a major role for the degradation of these compounds in the halogen cycle. As oxygen was not

available for the first 2 billion years on earth, oxygenolytic dehalogenation could not occur until oxygen-producing photosynthesis evolved and an oxygen-rich atmosphere developed. Early evolution was restricted to pathways using electron acceptors other than oxygen, giving rise to the numerous groups of anaerobic respiring gram positive and negative bacteria. In the course of the evolution, some anaerobes have learned to utilize halogenated compounds as terminal electron acceptors for anaerobic respiration processes (organohalide respiration) (Holliger *et al*., 2003).

Table 1.3 Different categories of biological dehalogenation

Dehalogenation type	Dechlorination reaction
reductive dehalogenation	R-Cl + 2 [H] → R-H + H^+ + Cl^-
hydrolytic dehalogenation	R-Cl + H_2O → R-OH + H^+ + Cl^-
oxidative dehalogenation	R-CH_2-Cl + O_2 + 2 [H] → R-CHO + H^+ + Cl^-
dehydrohalogenation	R-CH_2-CHCl-R' → R-CH=CH-R' + H^+ + Cl^-
alkyltransfer dehalogenation	R-Cl + XH → R-X + H^+ + Cl^-; XH = FH_4, GSH, etc.
dichloroelimination	R-CHCl-CHCl-R' + 2 [H] → R-CH=CH-R' + 2 H^+ + 2 Cl^-

Organohalide respiring anaerobes are able to couple anaerobic reductive dehalogenation to energy metabolism. The halogenated compounds, e. g. tetrachloroethene, 3-chloro-4-hydroxy-phenylacetate, may serve as terminal electron acceptors for the oxidation of an electron donor such as hydrogen or an organic substrate (Wohlfahrt and Diekert, 1997). The anaerobic bacteria capable of organohalide respiration are phylogenetically diverse, as shown in Fig. 1.2.

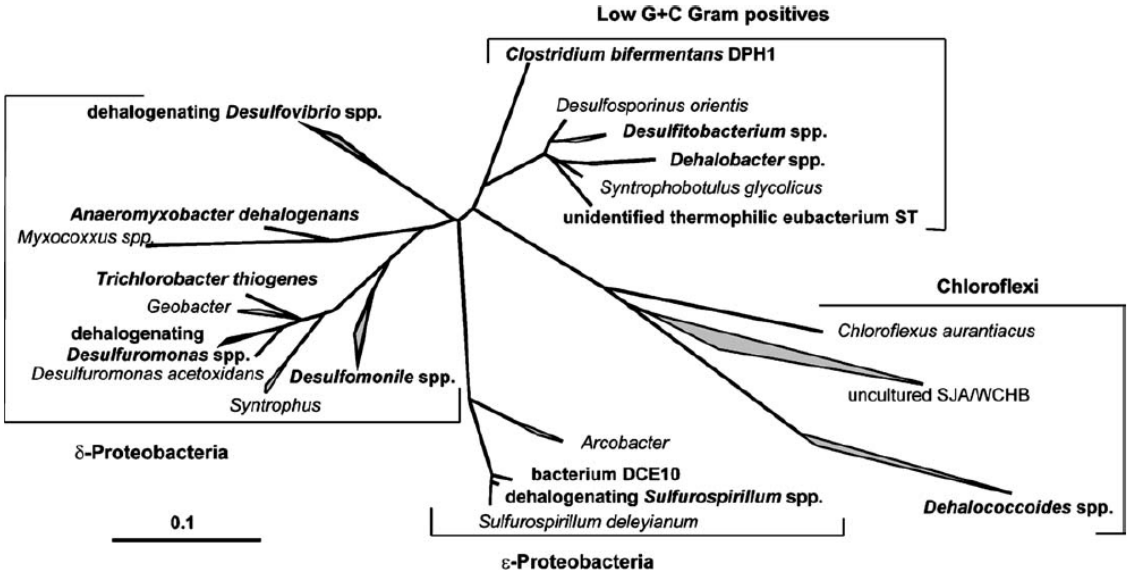

Fig. 1.2 Phylogenetic tree of organohalide respiring bacteria (in bold) based on bacterial SSU rRNA sequences (adapted from Smidt and de Vos, 2004).

A large variety of mono- and polyhalogenated organohalides have been reported to be dehalogenated by the organohalide respirers, ranging from chlorinated alkanes and alkenes, halogenated benzenes, and phenols to polychlorinated biphenyls and dioxins (Smidt and de Vos, 2004).

1.3.2 Reductive dehalogenases

Reductive dehalogenases are the key catalysts in the respiratory chain of organohalide respiring anaerobes. Several reductive dehalogenases with different substrate spectra have been purified and characterized from a number of organohalide respirers (Table 1.4). Except the 3-chlorobenzoate dehalogenase of *Dehalococcoides tiedjei*, all reductive dehalogenases described so far contain corrinoid and two iron-sulfur clusters as cofactors. All enzymes characterized so far are more or less tightly associated with the cytoplasmic membrane, supporting their role in membrane-associated electron transport-coupled phosphorylation.

Table 1.4 Reductive dehalogenases (RDs) purified from organohalide respiring bacteria

Reductive dehalogenase	Source	Reference
3-chlorobenzoate-RD	*Desulfomonile tiedjei* DCB-1	Ni *et al.*, 1995
Cl-OHPA-RD[1]	*Desulfitobacterium hafniense* DCB-2	Christiansen *et al.*, 1998
2,4,6-trichlorophenol-RD	*Desulfitobacterium frappieri* PCP1	Boyer *et al.*, 2003
3,5-dichlorophenol-RD	*Desulfitobacterium frappieri* PCP1	Thibodeau *et al.*, 2004
PCE-RD[2]	*Sulfurospirillum multivorans*	Neumann *et al.*, 1996
	Desulfitobacterium hafniense PCE-S	Miller *et al.*, 1998
	Dehalococcoides ethenogenes 195	Magnuson *et al.*, 1998
	Desulfitobacterium hafniense TCE1	van de Pas *et al.*, 2000
	Clostridium bifermentans DPH-1	Okeke *et al.,* 2001
	Desulfitobacterium sp. Y51	Suyama *et al*., 2002
	Dehalobacter restrictus	Maillard *et al.*, 2003
	Desulfitobacterium sp. strain KBC1	Tsukagoshi *et al*., 2006
TCE-RD[3]	*Dehalococcoides ethenogenes* 195	Magnuson *et al.*, 1998, 2000
DCE/VC-RD[4]	*Dehalococcoides* sp. strain VS	He *et al.*, 2003
	Dehalococcoides sp. strain BAV-1	Krajmalink-Brown *et al.*, 2004

[1]Cl-OHPA, 3-chloro-4-hydroxy-phenylacetate; [2]PCE, tetrachloroethene; [3]TCE, trichloroethene; [4]DCE, dichloroethene; VC, vinyl chloride.

Although different classes of non-reductive dehalogenases from aerobic bacteria such as haloalkane-, haloacid-, and 4-chlorobenzoyl-CoA dehalogenases have been studied extensively,

the study on dehalogenating enzymes from anaerobic microorganisms has just started since the last two decades. Hence, the substrate spectra and reaction mechanisms still need to be investigated in more details.

Although dechlorination has been studied for decades in various organisms (DeWeerd *et al.*, 1990; Madsen and Licht, 1992; Scholz-Muramatsu *et al.*, 1995), biological debromination has long been ignored. Just a few studies on biological debromination of halogenated aliphatic compounds exist. Different methanogenic archeae were reported to slowly dehalogenate dibrominated ethenes producing mainly acetylene and low amounts of ethene (Blay and Daniels, 1987). A mixed culture was described by Patterson *et al.* (2007) that dehalogenated brominated ethenes at a very low conversion rate: in a time period of more than 200 days about 2 µmol tribromoethene was debrominated to *cis*-1,2-dibromoethene (*c*DBE) and *trans*-1,2-dibromoethene (*t*DBE), about 1.5 µmol 1,2-DBE isomer mixture was dehalogenated to vinyl bromide and/or acetylene. These slow dehalogenation processes are presumably abiotic reactions mediated by corrinoids or coenzyme F_{430} as reported also for the conversion of halogenated methanes (Krone *et al.*, 1989a, b).

A few more reports are available on the dehalogenation of brominated aromatic compounds including brominated biphenyls (Morris *et al.*, 1992; Wu and Wiegel, 1997; Bedard and van Dort, 1998; Tokarz *et al.*, 2008), brominated bisphenols (Voordeckers *et al.*, 2002; Arbeli *et al.*, 2006), and brominated phenols (Steward *et al.*, 1995; Boyle *et al.*, 1999) or benzoates (Mohn and Tiedje, 1992).

However, most of the studies on debromination were performed with sediment, mixed or enrichment cultures. To get better understanding on anaerobic debromination catalyzed by reductive dehalogenases, studies should be carried out with purified enzymes from pure cultures.

1.4 Aims of the study

Most studies on halogenation as well as dehalogenation are focused on the cycling of chlorine. Little is known about the halogen reactions involving other halide ions. In this work the conversion of brominated alkenes by *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* strain PCE-S and their reductive dehalogenases is used as a model system to study debromination and this reaction is compared to the reductive dechlorination. In addition, bromination reactions cata-

lyzed by versatile peroxidases of *Bjerkandera adusta* strain Ud1 are studied as well as novel reactions of manganese peroxidases in the presence of fluoride are described.

The ability to produce halogenated compounds has been described to be ubiquitous among white rot fungi; these organohalogens might be released into the environments (de Jong *et al.*, 1994a) and part of them may become substrates for dehalogenating bacteria (Milliken *et al.*, 1997). Anaerobic zones are present in soil aggregates, even in aerated forest soils permanent anoxic micro-zones occur, and the anoxic environment in soils can be enhanced by intensive rainfall (Kuesel and Drake, 1996). Thus, the release and deposition of the fungal products into the environment might render these compounds available to anaerobic bacteria. Other fungal products are phenyl methyl ethers that are frequently produced during lignin degradation and have been reported to be electron donors supporting respiratory growth for some *O*-demethylating anaerobes; members of the genus *Desulfitobacterium* have even been described to utilize phenyl methyl ethers as electron donors for the reductive dechlorination of halogenated aromatic compounds (Neumann *et al.*, 2004). Therefore, a simple halogen cycle might exist in which lignin degrading aerobic fungi produce phenyl methyl ethers and halogenated aromatic compounds and anaerobic bacteria might utilize these fungal products for growth. A model system of a simplified halogen cycle is established in the laboratory using halogenating aerobic fungi and reductively dehalogenating anaerobic bacteria as the involved organisms.

Our aims are summarized as follows:

1. To shed some light on the halogenation and dehalogenation processes catalyzed by microorganisms and their enzymes involving halide ions (bromide, fluoride) other than chloride.
2. To find some evidence for the potential interaction between aerobic fungi which have the capacity of organohalogen and phenyl methyl ether production and anaerobic bacteria which are able to dehalogenate and *O*-demethylate.

2. Materials and Methods

2.1 Cultivation of microorganisms

2.1.1 Cultivation of anaerobic bacteria

The basal medium for anaerobic bacteria contained per liter: 0.2% (w/v) yeast extract, 0.07 g Na_2SO_4, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 1.0 g NaCl, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.5 g KCl and 0.15 g $CaCl_2 \cdot 2H_2O$. The complete medium was prepared by mixing 1 liter basal medium with 10 ml supplement solution, 40 ml 1 M $NaHCO_3$, 1 ml 5% (w/v) cysteine·HCl, 2.5 ml 36 mM $FeSO_4$ (in 50 mM H_2SO_4) and the substrates as stated in the Results section. The supplement solution consisted of vitamins and trace elements. The vitamins (5 mg cyanocobalamin, 4 mg *p*-aminobenzoic acid, 1 mg biotin, 10 mg nicotinic acid, 5 mg calcium pantothenate, 15 mg pyridoxamine·2HCl and 10 mg thiamin·HCl) and trace elements (1.6 mM HCl, 0.1 g $FeSO_4 \cdot 7H_2O$. 7 mg $ZnCl_2$, 10 mg $MnCl_2 \cdot 4H_2O$, 0.6 mg H_3BO_3, 13 mg $CoCl_2 \cdot 6H_2O$, 0.2 mg $CuCl_2 \cdot 2H_2O$, 2.4 mg $NiCl_2 \cdot 6H_2O$, 3.6 mg $Na_2MoO_4 \cdot 2H_2O$, 0.26 mg $Na_2SeO_3 \cdot 5H_2O$ and 0.66 mg Na_2WO_4) were dissolved in water and anaerobized and then added into 1 liter anaerobic/autoclaved 0.1 M potassium phosphate buffer (pH 7.5) via sterile filtration. $FeSO_4$, cysteine, $NaHCO_3$ as well as the supplement solution were anaerobized and autoclaved separately and added into the anaerobic/sterile basal medium with sterile syringes.

Sulfurospirillum multivorans (DSMZ 12446) (Scholz-Muramatsu *et al.*, 1995) and *Desulfitobacterium hafniense* strain PCE-S (DSMZ 14645) were routinely grown at 28°C in an anoxic medium with pyruvate (40 mM) or formate (40 mM) as electron donor and halogenated ethenes as electron acceptor (applied in the concentrations indicated in the Results section). When the cells were grown with formate, acetate (5 mM) was added as carbon source. *Desulfitobacterium hafniense* strain DCB-2 (DMSZ 10664; Madsen and Licht, 1992) was routinely grown at 37°C in an anoxic medium with pyruvate (40 mM) as electron donor and fumarate (40 mM) or halogenated aromatic compounds as electron acceptor.

Serum bottles sealed with Teflon-lined rubber septa were used for cultivation. The gas phase was 100% N_2 (150 kPa). The halogenated compounds were supplied in hexadecane as previously described (10 mM tetrachloroethene or 0.3 mM 1,2-dibromoethene or 5 mM 2,6-dichloroanisole) (John, Rubick *et al.*, 2009) or supplied as stock solution in ethanol (200 µM 3,5-dichloro-4-methoxybenzoic acid and 3-Cl-*p*-anisaldehyde in medium; final concentration of ethanol in medium < 1%). The medium was inoculated with 10% preculture as inoculum. The consumption and conversion of the halogenated substrates were monitored by gas chromatography (GC) (Section 2.6.1) or high performance liquid chromatography (HPLC) (Section 2.6.2).

2.1.2 Cultivation of fungi

Fungal strains were maintained on malt extract agar media (malt extract 20 g/l, glucose 5 g/l, peptone 1 g/l, agar 15 g/l). Fungal cultures were stored at 4℃. For haloperoxidase production, fungal strains were grown in a medium with high nitrogen content (50 mM N) supplied as peptone, according to Kimura *et al.* (1990) with minor modifications (Table 2.1). Medium (25 ml liquid volume) in a 250-ml serum bottle was sterilized at 121℃ for 30 min. Medium was inoculated with a plug (diameter 5 mm), which was taken from an agar medium covered with fresh mycelium of the fungal strain. Duplicate fungal cultures were incubated in the dark at 24℃. A duplicate set of sterile medium containing a sterile agar plug were incubated in parallel to the fungal cultures and were harvested as controls at the time of enzyme analysis. When the culture fluid was covered by the mycelium (2-6 weeks), the mycelia and the culture fluids were harvested for dry weight measurement and haloperoxidase assay, respectively.

Table 2.1 Composition of high nitrogen content medium

Chemical	g/l
glucose • H_2O	20.00
meat peptone	5.00
yeast extract	2.00
KH_2PO_4	1.00
$MgSO_4$ • $7H_2O$	0.50
NaCl or NaBr	0.06 or 0.12

For production of halogenating enzymes and halogenated compounds, *Bjerkandera adusta* strain Ud1 was cultivated in high nitrogen content medium or in beech wood chip semi-solid medium, where wood chips (>3 mm) were moistened with three portions (w/v) of distilled water or 1 mM NaCl or NaBr when indicated, and harvested at the time indicated in Results section.

2.1.3 Bacterial enrichment cultures

Enrichment cultures were derived from a forest soil, collected 8-10 cm below a spruce litter (Schwarzer Grund, Jena). The soil was transported in an aerobic flask flushed with nitrogen. Soil slurry was made with anaerobic bacterial medium described above (yeast extract was omitted) by stirring under anaerobic atmosphere overnight. It was then filtrated through filter paper to get rid of the soil particles. For the enrichment of *O*-demethylating/dehalogenating anaerobes, 5 mM 2,6-dichloroanisole (in hexadecane) was used as the sole electron donor and acceptor besides 0.02% (w/v) yeast extract. After four passages, the culture was transferred into a medium containing 20 mM vanillate as the electron donor and 6 mM 3-Cl-4-hydroxy-phenylacetate as the electron acceptor. For every passage, 10% inoculum was used. The conversion of the substrates and accumulation of the products was monitored with HPLC (see section 2.6.2).

Anaerobic roll tubes were used for isolation of the bacteria responsible for *O*-demethylation and dehalogenation from the enrichment culture. 20 mM vanillate was used as the electron donor and 6 mM 3-Cl-4-hydroxy-phenylacetate was the electron acceptor. The medium also contained 0.02% (w/v) yeast extract. Washed agar (2.5%) was used to solidify the medium. After 2-3 weeks cultivation, single colonies were picked up and transferred into 1 ml liquid medium containing the same substrates. A subculture into 25 ml medium was followed and the conversion of the substrates was monitored with HPLC.

2.2 Preparation of bacterial cell extracts, soil protein extracts and purification of enzymes

2.2.1 Preparation of bacterial cell extracts

S. multivorans and *D. hafniense* PCE-S cells were harvested in the late exponential growth phase by centrifugation (12,000 x g, 15 min at 10°C). The pellet was resuspended in 3 volumes (v/w) of 50 mM Tris-HCl (pH 7.5) and the cells were disrupted with a French Pressure Cell (Medium Cell, 1000 PSI, Thermo Electron Corporation, Waltham, USA). To avoid loss of membrane bound enzymes, the crude extracts were used for tribromoethene conversion without centrifugation.

2.2.2 Preparation of soil protein extracts

To check for ligninolytic enzyme activities in forest soil, soil proteins were extracted following Criquet and coworkers (1999) with minor modifications. 5 g of soil was added to 25 ml of 1 M $CaCl_2$ solution containing 0.05% Tween 80, and 20 g polyvinylpolypyrrolidone. The flask was shaken at room temperature for 1 h at 120 rpm. The suspension was centrifuged and filtered through 0.2µm filter and ultrafiltrated over 10 kDa membrane until the final volume was reduce to approximately 1/10 of the initial volume. The resulted extracts were used for manganese peroxidase, lignin peroxidase, laccase and Mn-independent peroxidase assays.

2.2.3 Purification of PCE dehalogeanse from *S. multivorans*

The tetrachloroethene (PCE) reductive dehalogenase from *S. multivorans* was purified as described by Neumann *et al.* (1996) with minor modifications. *S. multivorans* was grown anaerobically on a medium containing pyruvate and fumarate. The bacteria were harvested in the late exponential growth phase by centrifugation at 12,000 g and at 10°C for 15 min, frozen in liquid nitrogen, and stored frozen at -20°C under aerobic conditions.

Cell pellets of *S. multivorans* were resuspended in 50 mM Tris-HCl (pH 7.5; 3 ml/g pellet) containing 1 mM $MgCl_2$, 2 mM 4-(2-aminoethyl)-benzene-sulfofluoride, 10 mg/ml lysozyme as well as 1

mg/ml DNase I and were incubated for 1 h at 37°C. Cell debris was removed by ultracentrifugation under a N_2/H_2 (95%/5%; v/v) atmosphere for 1 h at 35,000 g and 4°C. The resulting supernatant was applied to a Q-Sepharose HP column (1.6×10 cm) preequilibrated with Tris-HCl buffer [50 mM Tris-HCl (pH 7.5) containing 0.5 mM 1,4-Dithio-D,L-threitol (DTT)]. The dehalogenase was eluted with a linear gradient from 0 to 1.0 M KCl in Tris buffer at a KCl concentration of approximately 0.2 M. Fractions containing dehalogenase were pooled, and 3.2 M $(NH_4)_2SO_4$ in Tris-HCl buffer was added to get a final concentration of 0.4 M. The solution was applied to a Phenyl-Superose HR column (1.0×10 cm) preequilibrated with 0.4 M $(NH_4)_2SO_4$ in Tris-HCl buffer. The enzyme was eluted with a linear gradient from 0.4 to 0 M $(NH_4)_2SO_4$ in Tris-HCl buffer at a $(NH_4)_2SO_4$ concentration of approximately 80 mM. Dehalogenase-containing fractions were pooled and applied to a Mono Q column (0.5×15 cm) preequilibrated with Tris-HCl buffer. The enzyme was eluted with a linear gradient from 0 to 0.5 M NaCl in Tris-HCl buffer at a NaCl concentration of approximately 0.2 M. All steps were performed in an anaerobic chamber with N_2/H_2 (95%/5%, v/v) as the gas phase.

2.2.4 Purification of PCE dehalogeanse from *D. hafniense* strain PCE-S

The PCE dehalogenase from *D. hafniense* PCE-S was purified as described by Miller *et al.* (1998) with minor modifications. *D. hafniense* strain PCE-S was grown anaerobically on a medium containing pyruvate and fumarate. The bacteria were harvested as described in Section 2.2.3.

Cell pellets of *D. hafniense* PCE-S were resuspended in 10 mM Tris-HCl (pH 7.5; 3 ml /g pellet) containing 0.5 mM DTT, 0.1% (v/v) Triton X-100 (reduced form), 2 mM 4-(2-aminoethyl)-benzene-sulfofluoride, 10 mg/ml lysozyme, 1 mg/ml DNase I and were incubated for 2 h at 37°C. After addition of EDTA and Triton X-100 (reduced form) to final concentrations of 1 mM and 0.15% (v/v), respectively, the cell extract was incubated at 4°C for 2 h. Cell debris was removed by ultracentrifugation under a N_2/H_2 (95%/5%; v/v) atmosphere for 1 h at 35,000 g and 4°C. The resulting supernatant was applied to a Q-Sepharose HP column (1.6×10 cm) preequilibrated with Mops buffer [50 mM Mops-KOH (pH 6.5) containing 0.5 mM DTT and 0.1% Triton X-100 (reduced form)]. The dehalogenase was eluted with a linear gradient from 0 to 0.5 M KCl in Mops buffer at a KCl concentration of approximately 0.1 M. Dehalogenase-containing fractions were pooled and applied to a Mono Q column (1.0×10 cm) preequilibrated with Mops buffer. The enzyme was eluted with a linear gradient from 0 to 0.5 M KCl in Mops buffer at a KCl concentration of approximately 0.1 M. All steps were performed in an anaerobic chamber with N_2/H_2 (95%/5%, v/v) as the gas phase.

2.2.5 Purification of brominating peroxidases from *B. adusta* strain Ud1

The brominating peroxidases of *B. adusta* strain Ud1 were purified as follows. The culture supernatant was separated from the mycelia by filtration through a 1.2-mm-pore-size filter and con-

centrated by ultrafiltration above a 10-kDa-membrane with an Amicon TCF10 ultrafiltration cell (Amicon Corp., Lexington, USA). The resulting extracellular proteins were applied to a Q-Sepharose HP column (1.6×10 cm) preequilibrated with 10 mM acetate buffer (pH 5.6). The brominating peroxidases (BrPOs) were eluted with a linear gradient from 0 to 1 M NaCl in acetate buffer at a NaCl concentration of approximately 0.3 M (BrPO-1) and 0.2 M (BrPO-2), respectively. The BrPO containing fractions were pooled separately and each was applied to a Mono Q column (0.5×5 cm) preequilibrated with 10 mM acetate buffer (pH 5.6). The enzymes were eluted with a linear gradient from 0 to 1.0 M NaCl in acetate buffer (pH 5.6) at a NaCl concentration of approximately 0.3 M and 0.2 M, respectively. For the purification of the BrPO from wood culture fluid (BrPO-3), the program of Q-Sepharose Chromatography was essentially the same, with the enzyme eluting at a NaCl concentration of 0.26 M. Instead of a Mono Q column, a Phenyl-Superose HR column (0.5×5 cm) was used as the next step. With a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$ in 10 mM acetate buffer (pH 5.6), the enzyme was eluted at a $(NH_4)_2SO_4$ concentration of approximately 0.74 M.

2.2.6 Purification of manganese peroxidase from *B. adusta* strain Ud1

The manganese peroxidase (MnP) of *B. adusta* strain Ud1 was purified in a similar way to that of BrPOs. After filtration and ultrafiltration, the culture supernatant was applied to a Q-Sepharose HP column (1.6×10 cm) preequilibrated with 10 mM acetate buffer (pH 5.6). The MnP was eluted with a linear gradient from 0 to 1 M NaCl in acetate buffer at a NaCl concentration of approximately 0.26 M. Fractions containing MnP were pooled and applied to a Mono Q column (0.5×5 cm) preequilibrated with 10 mM acetate buffer (pH 5.8). MnP was eluted with a linear gradient from 0 to 1.0 M NaCl in acetate buffer at a NaCl concentration of approximately 0.15 M. The MnP containing fractions were pooled and applied to a second Mono Q column (0.5×5 cm) preequilibrated with 10 mM acetate buffer (pH 5.8). Two MnP isoenzymes were eluted at this step and were named as MnP-1 and MnP-2 according to their order of elution. MnP-1 was eluted with an isocratic step of 0.1 M NaCl in acetate buffer. For further purification, the positive fractions were pooled and applied to a Phenyl-Superose HR column (0.5×5 cm). With a linear gradient of 1.0 to 0 M $(NH_4)_2SO_4$ in 10 mM acetate buffer (pH 5.6), the enzyme was eluted at a $(NH_4)_2SO_4$ concentration of approx. 0.3 M.

2.3 Enzyme assays

2.3.1 Reductive dehalogenation assay

The enzymatic activity of the PCE dehalogenases was measured photometrically by the oxidation of Ti(III) citrate-reduced methyl viologen (MV), which was applied as artificial electron donor for

the reduction of the halogenated ethenes (Neumann *et al.*, 1995) according to the equation:

$$R\text{-}Hal + 2\ MV^{+} + H^{+} \rightarrow R\text{-}H + Hal^{-} + 2\ MV^{2+}$$

MV oxidation was routinely recorded at 578 nm (ε_{578} = 9.7 $mM^{-1}cm^{-1}$) and 25°C in the presence of the enzymes and the halogenated compounds. If not otherwise stated, the assay was performed in 1 ml 100 mM Tris-HCl (pH 7.5) containing 0.5 mM MV and 0.7 mM halogenated hydrocarbon in rubber-stoppered glass cuvettes with N_2 (120 kPa) as the gas phase. Where indicated, 4 mM $(NH_4)_2SO_4$ was added to the assay. The reaction was started by the addition of enzyme. The rates are given as mol halide released corresponding to 2 mol MV oxidized per second (kat).

The kinetic constants of the PCE dehalogenases were determined using the spectrophotometric assay described above by varying the concentrations of halogenated ethenes at a MV concentration of 1.6 mM in the assay (Neumann *et al.*, 1996). The assay contained 4 mM ammonium sulfate, when the enzyme of *S. multivorans* was analyzed. About 50% of the MV was reduced by Ti(III) citrate prior to the addition of substrate. The reaction was started by the addition of enzyme. It was monitored at 700 nm (ε_{700} = 2.31 $mM^{-1}cm^{-1}$). The initial rate of the absorption decrease was used for the calculation of the activity. The data for the enzyme kinetics were fitted to Michaelis-Menten kinetics (assuming substrate inhibition, where indicated), and the apparent K_m and K_i values were calculated using a computer program (GraphPad Prism; GraphPad Software, San Diego, CA, USA).

For the enzymatic tribromoethene (TBE) conversion, essentially the same assay was used (0.8 µmol TBE in 1 ml 100 mM Tris-HCl buffer pH 7.5 with 10 mM MV; 12 ml flasks, gas phase: 100% N_2 or H_2, 1 atm; T = 25°C). The MV was reduced by addition of Ti(III) citrate or by the hydrogenase of the organism in the presence of H_2. Crude extract (0.6 mg protein) was added at t = 0 min. For each measurement, a different flask was used. At the times indicated in the Results section, the reaction mixtures were sacrificed, and samples were taken from the aqueous phase and analyzed for brominated ethenes by gas chromatography (see Section 2.6.1).

To test for a potential radical mechanism of the dehalogenation reaction, the assay described above was modified essentially according to Schmitz *et al.* (2007). The oxidation of 0.5 mM MV upon reduction of about 100 µM (initially) of the halogenated hydrocarbons was followed photometrically at 700 nm. When the reaction stopped due to the limiting concentration of the electron acceptor, Ti(III) citrate was added repeatedly to completely re-reduce oxidized methyl viologen. The reduction of MV was monitored again at 700 nm.

2.3.2 Fungal halogenating enzyme assays

All fungal enzyme activities were measured as initial velocities taking linear increments (or decrease in monochlorodimedone assay) at 25ºC using a Cary 100 Bio UV/visible spectrophoto-

meter (Varian, Darmstadt, Germany).

Halogenating activity was routinely assayed with monochlorodimedone (MCD) as substrate in 100 mM citrate/phosphate buffer (pH 2.8), as previously described (Hager *et al.*, 1966) (Fig. 2.1a). If not otherwise stated, 20 mM fluoride, chloride or bromide or 100 µM iodide, was supplied in their sodium salt form. 2 mM hydrogen peroxide was used to start the reaction. Kinetics were recorded at 278 nm for the decrease of monochlorodimedone absorption (ε_{278} = 12.2 $mM^{-1}cm^{-1}$). For screening of halogenating enzyme activity in culture fluids as well as in fractions eluted during enzyme purification, phenol red assay was applied as an alternative (Fig. 2.1b). Phenol red at a concentration of 40 µM was used as substrate and 100 mM NaBr together with 2 mM hydrogen peroxide were present as the halogenating agent. The reaction was also performed in 100 mM citrate/phosphate buffer (pH 2.8). The formation of bromophenol blue was monitored at 592 nm.

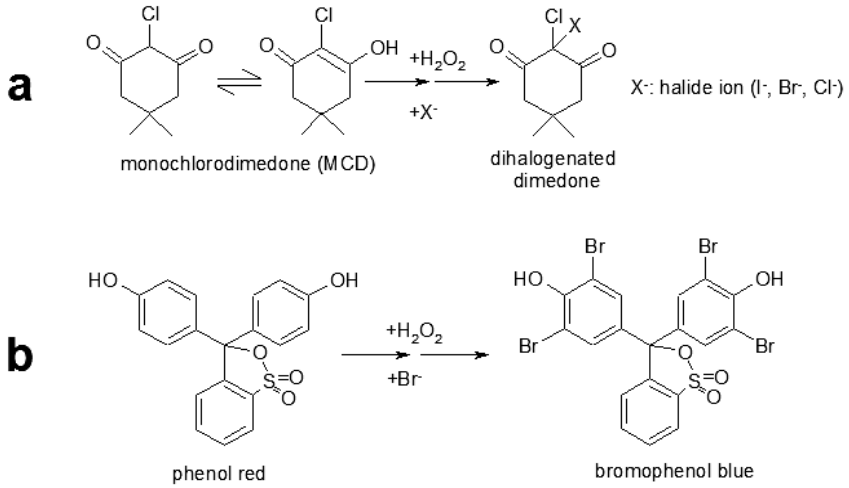

Fig. 2.1 Halogenating enzyme assays. a. monochlorodimedone assay; b. phenol red assay.

Using the spectrophotometric assay described above, the pH optima for bromination with monochlorodimedone (0.1 mM) as substrate were determined in 100 mM citrate/phosphate buffer at pHs from 2.0 - 8.0 in the presence of 10 mM NaF. H_2O_2 (2.0 mM) was used to start the reactions. Enzyme kinetic constants (apparent K_m and K_i) for bromide and hydrogen peroxide were determined in 100 mM citrate/phosphate buffer (pH 2.8). If not otherwise stated, 20 mM NaF and 1 mM H_2O_2 was used. The apparent K_m and K_i values were calculated using GraphPad Prism.

Bromination of phenol was performed in 1 ml 20 mM potassium phosphate buffer (pH 3.0) containing 0.5 mM phenol, 10 mM NaBr and 5 µg brominating enzyme. Reaction was started by addition of 0.1 mM H_2O_2. Stepwise 100 µl was removed from the reaction mixture and the reaction was stopped by adding 2 µl of 37% HCl. The reaction mixture aliquots were analyzed on HPLC (see Section 2.6.2).

2.3.3 Ligninolytic enzyme assays

Manganese peroxidase was assayed at pH 4.5 in the presence of 50 mM malonate, 0.5 mM $MnCl_2$ and 0.2 mM H_2O_2. The formation of the Mn(III) malonate complex (ε_{270} = 11.59 $mM^{-1}cm^{-1}$) was followed. Lignin peroxidase activity was calculated from the oxidation of veratryl alcohol to veratraldehyde (ε_{310} = 9.3 $mM^{-1}cm^{-1}$). The reaction was performed in 100 mM tartrate buffer (pH 3.0) containing 4 mM veratryl alcohol and 0.2 mM H_2O_2. Laccase and manganese-independent peroxidase activities were measured from ABTS (0.3 mM) oxidation (ε_{420} = 36.0 $mM^{-1}cm^{-1}$) in 100 mM tartrate buffer (pH 4.5) in the absence and presence of 0.2 mM H_2O_2, respectively.

The pH optima and enzyme kinetic constants (apparent K_m and K_i) were determined for veratryl alcohol oxidation and/or Mn(II) oxidation using the spectrophotometric assays described above. If not otherwise stated, 4 mM veratryl alcohol in 100 mM tartrate buffer (pH 3.0) or 0.5 mM $MnCl_2$ in 50 mM malonate buffer (pH 4.5) was present and 0.2 mM H_2O_2 was used to start the reactions. The initial rate of the absorption decrease was used for the calculation of the activity. The apparent K_m and K_i values were calculated using GraphPad Prism.

2.3.4 Assays for fluoride-dependent reactions mediated by MnP-1 of *B. adusta* strain Ud1

MnP-1 mediated conversion of 0.1 mM monochlorodimedone (ε_{278} = 12.2 $mM^{-1}cm^{-1}$), 1 mM 2,6-dimethoxyphenol (ε_{469} = 27.5 $mM^{-1}cm^{-1}$), 1 mM guaiacol (ε_{470} = 26.6 $mM^{-1}cm^{-1}$), 0.3 mM ABTS (ε_{420} = 36 $mM^{-1}cm^{-1}$) or 4 mM veratryl alcohol (ε_{310} = 9.3 $mM^{-1}cm^{-1}$) was preformed in 100 mM citrate/phosphate buffer (pH 2.8) in the presence of 20 mM NaF and 1 mM H_2O_2. Oxidation of 1,2,3,5-tetramethoxybenzene (0.2 mM) was monitored at 275 nm in the presence of 20 mM NaF and 0.2 mM H_2O_2.

Using the spectrophotometric assay described above, the pH optima for fluoride-dependent reactions with monochlorodimedone (0.1 mM) or 2,6-dimethoxyphenol (1.0 mM) as substrates were determined in the absence of Mn(II) in 100 mM tartrate buffer at pHs from 2.0 - 8.0 in the presence of 10 mM NaF. 2.0 mM H_2O_2 was used to start the reactions. The pH optimum for Mn(II) (0.5 mM) oxidation was also determined in 100 mM tartrate buffer at pHs from 2.0 - 8.0. H_2O_2 (0.2 mM)was added to start the reactions.

Enzyme kinetic constants (apparent K_m and K_i) were determined for fluoride, hydrogen peroxide, monochlorodimedone (MCD) or 2,6-dimethoxyphenol in fluoride-dependent reactions, as well as for Mn(II) and hydrogen peroxide in Mn(II) oxidation using the spectrophotometric assays described above. If not otherwise stated, the MnP-1 mediated fluoride-dependent reactions were performed in 100 mM citrate/phosphate buffer (pH 2.8) in the presence of 20 mM NaF and 1 mM H_2O_2; whereas Mn(II) oxidation reaction was carried out in 50 mM malonate buffer (pH 4.5) con-

taining 0.5 mM Mn(II) and 0.2 mM H_2O_2. The initial rate of the absorption increase or decrease (MCD assay) was used for the calculation of the activity. The apparent K_m and K_i values were calculated using GraphPad Prism.

2.4 Characterization of the enzymes

2.4.1 SDS-PAGE

Proteins can be separated according to their size by SDS-PAGE (Ausubel *et al.*, 1987). The gels were prepared as following, using the apparatus from Biometra (Göttingen, Germany). Separation gel was prepared with 410 mM Tris-HCl pH 8.8, 0.1% SDS, 12-15% acrylamide (Rotiphorese® Gel 30; Roth, Karlsruhe), 2.7 mM TEMED and 4.4 mM APS. The polymerization of acrylamides and bisacrylamides with TEMED started upon addition of APS. After the separation gel was polymerized, the stacking gel was prepared with 58 mM Tris-HCl pH 6.8, 0.05% SDS, 5% acrylamide (Rotiphorese® Gel 30), 6.4 mM TEMED and 4.4 mM APS. For electrophoresis, the discontinuous Laemmli-System was used (Laemmli, 1970). The running buffer contained 25 mM Tris, 100 mM glycerol and 0.1% SDS. The protein samples were mixed with one volume of loading buffer [125 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerine, 10% (v/v) mercaptoethanol and 0.01% (w/v) bromophenol blue] and denaturized at 95°C for 5 min. The electrophoresis was performed at a current at 12 mA (stacking gel) or 25 mA (separation gel) per gel.

After the gel electrophoresis, the SDS gels were fixed in 25 % (v/v) isopropanol and 10 % (v/v) acetic acid for 15 min and stained with staining solution [240 mg/l Coomassie Brillant Blue G250 in 10% (v/v) acetic acid] for 20-30 min. For destaining of the background, the gels were developed in 10% (v/v) acetic acid.

2.4.2 Western blot

For western blot analysis, 1 µg protein of *S. multivorans* was separated on SDS-PAGE (15%) and subsequently blotted onto a PVDF-membrane (Ausubel *et al.*, 1987) with a semi-dry blot apparatus (Biorad, München) (15 V, 1 h). The membrane and the SDS gel were equilibrated in blotting buffer (25 mM Tris, 100 mM glycine, 20% (v/v) methanol) for 30 min before blotting. After the proteins were transferred onto the membrane, the unoccupied space of the membrane was blocked by 1 h incubation in 1% Western-Blocking-Solution (Roche, Mannheim) in PBST [140 mM NaCl, 10 mM KCl, 6.4 mM Na_2HPO_4, 2 mM KH_2PO_4 and 0.05% (v/v) Tween 20]. The membrane was then washed 3 x 10 min in PBST and incubated with the purified antibody (1:50000 in PBST) overnight at 18°C. On the second day, the membrane was washed 3 x 10 min in PBST and incubated with the secondary antibody [anti-rabbit-antibody, coupled with alkaline phosphatase (Biorad,

München); diluted 1:3000 in PBST] at room temperature for 1 h. After washing in PBST (3 x 10 min), the membrane was equilibrated in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ for 2 min. The alkaline phosphatase reaction was preformed in the same buffer by addition of the substrate (0.34 mg NBT and 0.175 mg X-phosphate per ml buffer). The reaction was stopped after 10 min with 10 mM Tris-HCl pH 7.4 and 1 mM EDTA.

The protein markers on the blotting membrane were stained in 0.2% (w/v) Coomassie® Brilliant Blue G250, 45% (v/v) methanol and 10% (v/v) acetic acid for 5 min. By washing in 90% (v/v) methanol with 7% (v/v) acetic acid the background where no protein was bound was destained and the marker bands became visible.

2.4.3 UV/VIS spectroscopy of fungal peroxidases

UV/VIS spectra of the purified fungal peroxidases were recorded on a Cary 100 Bio UV/visible spectrophotometer (Varian, Darmstadt, Germany) at room temperature using quartz cells of 1 cm path length. The concentrations of the proteins were around 1 µM in 10 mM acetate buffer (pH 5.6).

2.4.4 MALDI-TOF analysis of fungal peroxidases

The brominating peroxidases of *B. adusta* strain Ud1 were analyzed with matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF-MS in the Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology (Jena).

About 2 µg/lane of each brominating peroxidase was run on a 15% SDS-PAGE gel. Protein bands were excised from the gel and in-gel digested with trypsin according to the “In-gel digest (Coomassie stained) with trypsin” protocol released by Bruker Daltonik GmbH (Bremen, Germany). The gel particles were washed with water and 50 mM NH_4HCO_3/acetonitrile (1:1, v/v) for 15 min each and were dehydrated by addition of acetonitrile, swollen by rehydration in 50 mM NH_4HCO_3, and shrunk again by addition of the same volume of acetonitrile. Acetonitrile was then removed and the gel particles were dried in a vacuum centrifuge. A volume of 10 mM dithiotreitol (DTT) in 50 mM NH_4HCO_3 (freshly prepared) sufficient to cover the gel pieces was added, and the proteins were reduced for 45 min at 56°C. After cooling to room temperature, the DTT solution was replaced with roughly the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 (freshly prepared). After 30 min incubation at room temperature in the dark, the gel pieces were washed 2 × 15 min with 50 mM NH_4HCO_3/acetonitrile (1:1, v/v), dehydrated by addition of acetonitrile and dried in a vacuum centrifuge. The gel pieces were swollen in a freshly prepared digestion buffer containing 25 mM NH_4HCO_3 and 10 ng/µl of trypsin in an ice-cold bath. After 30 min, the supernatant was removed and replaced with 2-3 µl of 25 mM NH_4HCO_3 to keep the gel pieces wet during enzymatic cleavage (37°C, overnight). Peptides were extracted with acetonitrile/0.1% trifluoroacetic acid (TFA) (1:1, v/v)

for 60 min at room temperature with thorough mixing from time to time. The peptide sample (1 µl) was mixed with one volume of matrix solution (HAAC, solvent: 1/3 acetonitrile plus 2/3 0.1% TFA solution) and deposited on an anchor position. After the solvent evaporated, the proteins were analyzed on a Bruker Ultraflex I MALDI-TOF/TOF device (Bruker Daltonics, Bremen, Germany). The MS-spectra obtained were subsequently identified by searching the NCBI-database using the MASCOT interface. Some peptides were fragmented further (LIFT) to obtain MS/MS spectra.

2.4.5 N-terminal sequencing of fungal peroxidases

The N-terminal amino acid sequence determination of the fungal peroxidases with brominating activities was carried out in the Department of Biochemistry, Leibniz Institute for Age Research (Jena).

The purified brominating peroxidases (about 2 µg per lane) were separated by 15% SDS-PAGE and transferred to a PVDF-membrane. After staining with Coomassie Brilliant Blue G-250, the protein bands of interest were excised and the first 25 N-terminal amino acids were determined by Edman degradation with a protein sequencer (model 494A Procise, Applied Biosystems, Foster City, CA, USA).

2.4.6 Inactivation of *B. adusta* strain Ud1 MnP-1 in the absence of substrate

Inactivation of *B. adusta* strain Ud1 MnP-1 by hydrogen peroxide in the absence or presence of fluoride or Mn(II) was investigated in the absence of substrates. All reactions (1 ml) were performed in 100 mM citrate/phosphate buffer (pH 4.0) containing 3 µg MnP-1, 0.1 mM H_2O_2 and 20 mM NaF or 0.5 mM $MnCl_2$. Stepwise 50 µl was removed from the inactivation mixture and added to a 1-ml Mn(II)-malonate reaction mixture, which contained 50 mM malonate (pH 4.5), 0.5 mM Mn(II) and 0.2 mM H_2O_2, to start Mn(II) oxidation. The initial velocities of Mn(III)-malonate formation were taken for the calculation of MnP activity (ε_{270} = 11.59 $mM^{-1}cm^{-1}$).

2.4.7 Mn(II) or F^- binding to MnP-1 of *B. adusta* strain Ud1

Fluoride or manganese binding was studied using reference cuvettes and sample cuvettes, as described previously (Renganathan *et al.*, 1987). Both the reference and sample cuvettes contained 1 µM manganese peroxidase in 100 mM citrate/phosphate buffer (pH 3.4, 4.0 or 4.4). Aliquots of F^- or Mn(II) were added to the sample cuvettes, whereas the same volume of bidistilled water was added to the reference cuvettes. Absorption spectra were recorded from 550 to 350 nm after each addition of F^- or Mn(II) (or water in case of reference cuvettes) and difference spectra were obtained by subtracting the spectra of the reference cuvettes from those of the sample cuvettes. The apparent dissociation constants K_D were calculated from plots of $[F^-]^{-1}$ or $[Mn^{2+}]^{-1}$ vs.

$\triangle A^{-1}$ (the difference between maximum and minimum absorptions).

For competitive inhibition of Mn(III)-malonate formation by fluoride, tests were performed as described by Youngs *et al.* (2000) with minor modifications. The effect of fluoride on the oxidation of Mn(II) by MnP-1 was determined by following the formation of Mn(III)-malonate at 270 nm. Reactions were performed with 0.6 µg MnP I of *B. adutsa* strain Ud1 in 50 mM malonate buffer (pH 4.0) with Mn(II), hydrogen peroxide and fluoride concentrations given in Results section.

2.5 Identification of substances

GC-MS, LC-MS and ^{1}H NMR were performed in collaboration with the Department of Bioorganic Chemistry, Max-Planck Institute for Chemical Ecology (Jena).

2.5.1 Separation and identification of dibromoethenes

Since the individual isomers were not commercially available, a mixture of *cis*-DBE and *trans*-DBE was used in all experiments. To interpret the results correctly, it was necessary to separate and identify these two isomers. For this purpose, GC-MS and ^{1}H NMR were performed.

A baked out (250°C, He) SPME-fibre [PDMS, red, 100 µm film thickness, Sigma-Aldrich (Steinheim, Germany)] was held for 2 seconds into the headspace of the sample (about 1 ml sample in a 10 ml glass vial). Immediately after volatile collection the SPME-fibre was inserted into an Optic 3 injector (ATAS GL, Eindhoven, Netherlands) installed on a HP 6890 GC (Agilent, Santa Clara, CA, USA) equipped with a 30 m DB-1 column (ZB-1MS, 30 m x 0.25 mm, film thickness 0.25 µm; Phenomenex, Torance, CA, USA) coupled to a time-of-flight mass spectrometer (GCT, Micromass, Manchester, UK). Helium was used as carrier gas (1 ml/min); injector temperature: 220°C; split: 1:30, 1:200; GC oven temperature: 50°C (10 min). Mass spectra were recorded in positive EI mode (70 eV).

^{1}H NMR spectra were recorded on a Bruker Avance DRX 500 NMR spectrometer (500 MHz) (Bruker, Karlsruhe, Germany). Chemical shifts were reported in ppm from tetramethylsilane with the solvent resonance as the internal standard ($CHCl_3$: δ 7.26 ppm). ^{13}C NMR spectra were recorded on a Bruker Avance DRX 500 NMR spectrometer (125 MHz) with complete proton decoupling. Chemical shifts are given in ppm from tetramethylsilane with the solvent as the internal standard ($CDCl_3$: δ 77.0 ppm).

Two peaks were trapped from the synthetic mixture of *cis*- and *trans*-1,2-dibromoethene, and each was identified as dibromoethene by mass spectrometry. The two isomers in the synthetic mixture were identified as follows: The *cis* isomer has a higher boiling point than the *trans* isomer (*c*DBE: 112.5°C; *t*DBE: 108°C); hence, it should elute later from the GC capillary column used

(3.12 versus 2.75 min) (Fig. 2.2a). The ratio of the mixture according to GC peak integration (assuming the same sensitivity of the GC signal for both isomers) is *c*DBE/*t*DBE = 64/36; this ratio is also reflected in the integrals of the ^{1}H-NMR signals. The *cis* isomer was expected to show a more downfield resonance than the *trans* isomer by nuclear magnetic resonance spectroscopy (Hur *et al.*, 1994) and the major isomer was downfield (δ 7.0 ppm versus 6.6 ppm) (Fig. 2.2b), which allowed an unambiguous identification of the two isomers.

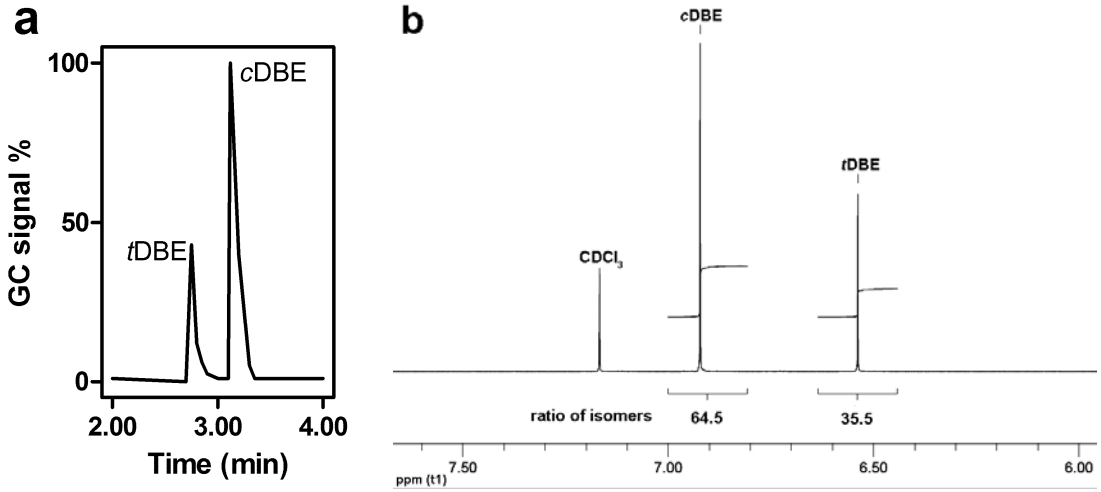

Fig. 2.2 Assignment of *cis*- and *trans*- dibromoethene in the standard mixture from Sigma-Aldrich. a. separation of *c*DBE and *t*DBE by gas chromatography; b. ^{1}H-NMR spectra of the *c*DBE and *t*DBE mixture.

2.5.2 Identification of fungal halogenated metabolites

The halogenated compounds produced by *B. adusta* strain Ud1 were extracted from the cultures with ethyl acetate and analyzed using gas chromatography/mass spectrometry on a Thermo-Finnigan TRACE GC/MS system (Thermo/Finnigan, Manchester, UK) equipped with a 15 m ZB-5 column with 10 m guardian end (ZB-1MS, 15 m x 0.25 mm, film thickness 0.25 µm; Phenomenex, Torance, CA, USA). Helium was used as carrier gas (1.5 ml/min); injector temperature: 220°C; split: 1:10; GC oven temperature: 70°C (2 min) followed by steps of 8°C increase to 250°C and then a step of 30°C increase to 280°C (2 min). Mass spectra were recorded in positive EI mode (70 eV).

2.5.3 Identification of the reaction products of MnP-1 mediated 2,6-dimethoxyphenol conversion

The reaction products of MnP-1 mediated 2,6-dimethoxyphenol conversion were extracted from the reaction mixture with dichloromethane and analyzed on a Dionex 3000 HPLC System equipped with a Phenomenex Gemini 5 µm HPLC column (250 mm x 2 mm; Phenomenex, Torance, CA,

USA) coupled with a Finnigan LTQ ESI-MS (Thermo Fisher Scientific, Waltham, MA, USA) (mass range:100 – 2000 Da). The mobile phase was composed of A: 0.5% AcOH in water and B: 0.5% AcOH in MeCN. After 3 min equilibration in 0.5% AcOH in water, a linear gradient of MeCN (0.5% AcOH) from 0% to 100% within 24 min was applied. The flowrate was 200 µl/min. The products were then analyzed with High Resolution-Mass Spectrometry using an LTQ-Orbitrap from Thermo-fisher at a resolution of 77000.

2.6 Analytical procedures

2.6.1 Gas chromatography (GC)

The halogenated aliphatic compounds were routinely quantified gas chromatographically by flame ionization detection (FID) using a 10% Ucon LB on WAW packed column (length 2 m, OD 6 mm, ID 2 mm; WGA Analysentechnik, Düsseldorf, Germany) and N_2 as carrier gas. The temperatures were as follows: oven, 80°C; injector, 150°C; detector, 250°C. The carrier gas flow was 25-30 ml per min at 300 kPa. The retention times under these conditions were: *c*DCE, 1.1 min; TCE, 1.6 min, PCE, 2.4 min; ethene, 0.38 min; VB, 0.57 min; *t*DBE, 2.6 min; *c*DBE, 3.7 min; 1,1-DBE, 1.4 min; 2,3-DCP, 2.8 min; 2,3-DBP, 5.6 min. DBE concentrations were calculated assuming that the sensitivity of the FID was the same for all three DBE isomers. If not otherwise stated, 1 ml sample was taken from the aqueous phase to a 12-ml glass vial stoppered with teflon-septa and heated at 95°C for 6 min before injecting the gas phase into the injector.

Due to the low solubility and high toxicity, the halogenated ethenes were supplied in hexadecane solutions as an overlay of the medium to the bacteria. In this way, a three phase system consisting of gas, hexadecane and medium was formed. To simplify measurements, the Henry's coefficient of each compound was determined (Table 2.2). 500 µmol of each halogenated compounds dissolved in hexadecane was supplied into 100 ml medium in 250 ml serum bottle stoppered with Teflon septa. The gas phase was 100% N_2 (150 kPa). Ethene (100 µmol) was directly supplied to the medium. All bottles were shaken at 28°C overnight. Samples were taken for GC analysis from gas phase, hexadecane phase and water phase. All measurements were done in duplicate or triplicate. In this way, the amount of substrates and products in the bacterial cultures could be calculated from the gas chromatography measurement of aqueous phase samples.

Table 2.2 Henry's Law coefficients in gas-hexadecane-water three-phase system

Substance	ethene	VB	*c*DBE	*t*DBE
water : hex : gas	1.00 : 0.08 : 7.80	1.00 : 0.12 : 0.44	1.00 : 1.02 : 0.10	1.00 : 0.45 : 0.05

[1]hex, hexadecane.

2.6.2 High performance liquid chromatography (HPLC)

A High Performance Liquid Chromatography (HPLC) system was used for monitoring the substrate conversion and/or product accumulation during halide ion-involved reactions catalyzed by fungal peroxidases. The system was equipped with a Lichrosphere 100 RP-18 column (Merk, 250×4 mm) and consisted of a Merk-Hitachi L-7100 pump, a Merk-Hitachi L-7200 autosampler and a Merk-Hitachi L-4200 UV/VS detector. The production of halogenated aromatic compounds by *B. adusta* strain Ud1 and the conversion of these organohalogens by *D. hafniense* DCB-2 were also analyzed on HPLC. The details of the HPLC methods used are listed in Table 2.3.

Ethyl acetate was used for extraction of halogenated compounds produced by *B. adusta* strain Ud1. Dichloromethane was applied to extract the reaction products of MnP-1 mediated fluoride-dependent monochlorodimedone or 2,6-dimethoxyphenol conversion.

2.6.3 Quantification of bacterial growth

The bacterial growth was denoted by protein concentrations measured in disrupted cells. Cells were collected at different time points by centrifugation and subsequently disrupted by 95°C heating in the presence of 1/5 volume 0.2 M NaOH for 5 min. After centrifugation, the supernatants were taken for protein concentration determination with Bradford method (1976) using Roti-Nanoquant (Roth, Karlsruhe, Germany) as reagent according to the manufacturer's instructions. To make sure no interference on protein concentration determination was caused by the halogenated compounds dissolved in the medium, internal standards of bovine serum albumin were used in such cultures. No disturbing effect was observed.

2.6.4 Determination of hydrogen peroxide concentrations

Hydrogen peroxide consumption was monitored during enzyme inactivation. The H_2O_2 concentration was determined indirectly via ABTS oxidation in the presence of horseradish peroxidase. First, an incubation solution was prepared with 20 ml phosphate buffer (pH 7.0) together with 0.04 ml horseradish peroxidase (0.6 mg/ml) and 0.4 ml ABTS (25 mg/l). 10 µl sample (reaction stopped by addition of 1/50 volume of 37% HCl) was then incubated in 200 µl incubation solution for 20 min. The color changes due to the conversion of ABTS to $ABTS^+$ was recorded at 420 nm with a SpectraMax 340PC Microplate Spectrophotometer (Molecular Devices, Sunnyvale, USA) at room temperature using a 96-well microplate. A standard curve was achieved with H_2O_2 concentrations from 0 to 200 µM.

Table 2.3 Detailed methods of HPLC analysis. WL, wavelength; Inj., injection volume; DMP, 2,6-dimethoxyphenol; DCMBA, 3,5-dichloro-4-methoxybenzoic acid; DCHBA, 3,5-dichloro-4-hydroxybenzoate; CHBA, 3-Cl-4-hydroxybenzoate; DHB, 3,4-dihydroxybenzoic acid; ClOHPA, 3-Cl-4-hydroxy-phenylacetate; OHPA, 4-hydroxy-phenylacetate.

Substance	WL (nm)	Inj. (µl)	Mobile phase	Program	Retention time	Reference
phenol; 2-bromophenol; 4-bromophenol	254	20	A: 0.3% H_3PO_4 B: 100% methanol	40% A, 60% B; 0.5 ml/min	8.4 min; 13.9 min; 24.6 min	This work
monochlorodimedone	278	20	A: 200 mM K_3PO_4 buffer (pH 6.0), B: methanol	80% A, 20% B; 1 ml/min	7.0 min	Kataoka *et al.*, 2000
veratraldehyde; 3-Cl-*p*-anisaldehyde	265	30	A: water; B: 100% acetonitrile	0 min: 15% B; 10 min: 15% B; 20 min: 90% B; 24 min: 90% B; 0.8 ml/min.	11.6 min; 15.4 min	This work
2,6-dimethoxyphenol	280	20	A: 0.3% H_3PO_4; B: methanol	35% A, 65% B; 0.75 ml/min	4.3 min	This work
products of DMP conversion	469	10	A: 15% acetonitrile in 0.05% H_3PO_4; B: 100% acetonitrile	0 min: 100% A; 15 min: 100% B; 1 ml/min	7.9 min; 8.5 min	Wariishi *et al.,* 1992
DCMBA; DCHBA; CHBA	210	10	A: 0.3% H_3PO_4; B: methanol	50% A, 50% B; 0.8 ml/min	31.6 min; 12.3 min; 7.5 min	This work
2,6-dichloroanisole; 2,6-dichlorophenol; 2-chlorophenol	254	20	A: 0.3% H_3PO_4; B: methanol	35% A, 65% B; 0.5 ml/min	11.9 min; 5.9 min; 4.9 min	This work
vanillate; DHB; ClOHPA; OHPA	210	10	A: 0.3% H_3PO_4; B: methanol	72% A, 28% B; 0.8 ml/min	11.1 min; 6.1 min; 17.8 min; 8.4 min	This work

2.7 Identification of a bacterial strain isolated from forest soil

The identification of the bacterial strain was performed as described previously (Weisburg *et al.*, 1991). After genomic DNA extraction with PowerSoil™ DNA Isolation Kit (Mo Bio Labs, Carlsbad, California, USA), 16S ribosomal DNA was amplified with a eubacteria-specific primer pair (forward: 5'- agagtttgatcctggctcag-3' and reverse: 5'-acggc taccttgttacgactt-3') using GoTaq Polymerases Kit (Promega, Mannheim, Germany) on an Eppendorf Mastercycler® Personal Thermal Cycler. The reaction mixture (50 µl) contained 1.25 U GoTaq, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer and approximately 1 ng template DNA. The PCR program consisted of 25 cycles of 95°C (2 min), 42°C (30 s), and 72°C (4 min), plus one additional cycle with a final 20-min chain elongation. The PCR product was excised from the agarose gel (1‰ after electrophoresis. After extraction with QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), the PCR product was sent for sequencing at GATC Biotech AG (Konstanz, Germany). The 16S rDNA sequence was compared to the non-redundant collection (Genbank, DDBJ, EMBL & PDB) of sequences using BLAST on the website of NCBI (http://www.ncbi.nlm.nih.gov/).

2.8 Source of materials

Tribromoethene was purchased from Fluorochem (Derbyshire, UK). 2,3-dichloropropene and 2,6-dichlorophenol was obtained from Acros Organics (Nidderau, Germany). 3,3',5,5'-tetramethoxy-4,4'-diphenoquinone was from Dr. Ehrens torfer GmbH (Augsburg, Germany). Bromophenol blue, monochlorodimedone, 4-hydroxy-phenylacetate were supplied by Fluka (Steinheim, Germany). Veratraldehyde and 2-chlorophenol were obtained from Fluka (Buchs, Germany). 4-bromophenol, 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid were purchased from MERCK-Schuchardt (Munich, Germany). All other chemicals used were of the highest available purity and were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), Roche (Mannheim, Germany) and Roth (Karlsruhe, Germany). Gases (CO_2 grade 4.5, N_2 grade 5.0, N_2/H_2 95:5, v/v) were supplied by Linde (Leuna, Germany). Immunization and production of the PceA-antibody was done by SeqLab (Göttingen). The secondary antibody was provided by Biorad (Munich, Germany) or British Biocell International (Cardiff, UK). Chromatography media and instruments were from Pharmacia Biotech (Uppsala, Sweden).

3. Results

3.1 Reductive dehalogenation of brominated ethenes by *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* PCE-S

3.1.1 Reductive debromination of brominated ethenes in cultures

Sulfurospirillum multivorans and *Desulfitobacterium hafniense* PCE-S were precultured for at most 5 subcultures (10% inoculums) with pyruvate plus fumarate so that the organisms still contained a fully induced PCE dehalogenase (John, Rubick *et al.*, 2009). They were then transferred to media containing pyruvate (40 mM) as electron donor and 1,2-dibromoethene (1,2-DBE) as electron acceptor (64/36 mixture of *cis*- and *trans*-1,2-DBE; about 400 µmol 1,2-DBE in hexadecane corresponding to near 1 mM DBE in the medium). Both organisms were able to dehalogenate the brominated ethenes (Fig. 3.1).

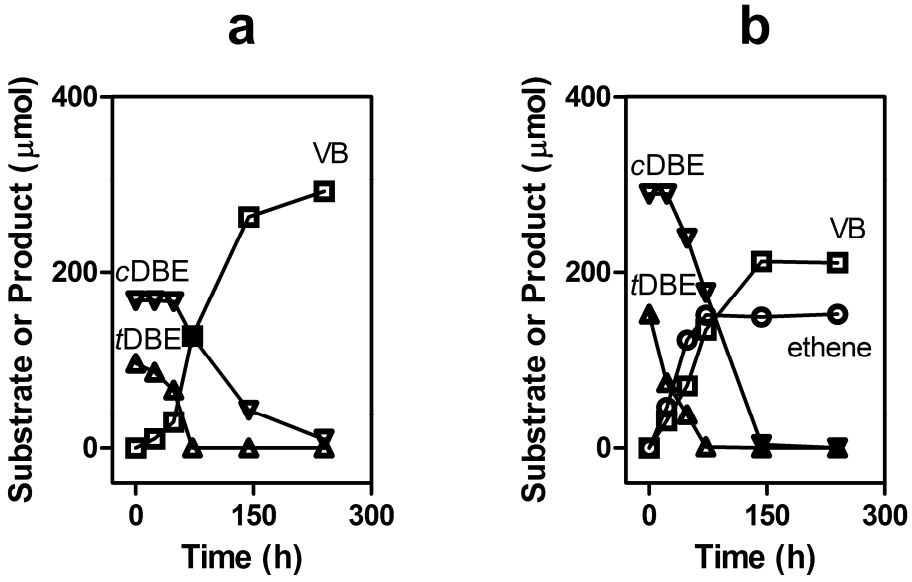

Fig. 3.1 Dehalogenation of dibromoethene (1,2-DBE) by cells of *S. multivorans* (a) and *D. hafniense* PCE-S (b) in media supplemented with 40 mM pyruvate. 1,2-DBE (64/36 mixture of *c*DBE/*t*DBE) was dissolved in hexadecane and supplied as an organic layer to the medium. Brominated ethenes and ethene were monitored in the aqueous phase by gas chromatography. ∆, *t*DBE, *trans*-1,2-DBE; ∇, *c*DBE, *cis*-1,2-DBE; □, VB, vinyl bromide; ○, ethene (b).

Both *S. multivorans* and *D. hafniense* strain PCE-S converted *trans*-1,2-dibromoethene (*t*DBE) prior to *cis*-1,2-dibromoethene (*c*DBE); only when *t*DBE was almost completely consumed, *c*DBE

conversion started. *S. multivorans* formed exclusively vinyl bromide (VB) as the debromination product, whereas cells of *D. hafniense* PCE-S also produced significant amounts of ethene under the conditions applied (Fig. 3.1).

The brominated ethenes at these concentrations (near 1 mM DBE in the medium) were inhibitory for growth (see also Fig. 3.2a). To examine growth of both organisms with 1,2-DBE as electron acceptor, the 1,2-DBE concentration was lowered. After adding 1,2-DBE stock solution of different concentrations in hexadecane to the medium, initial concentrations of about 80 µM, 150 µM, 190 µM or 250 µM were measured in the aqueous phase. For *S. multivorans*, 150 µM 1,2-DBE was already inhibitory, whereas growth was stimulated by 80 µM 1,2-DBE (Fig. 3.2a). *D. hafiense* PCE-S seemed to be even more sensitive to 1,2-DBE; as low as 80 µM 1,2-DBE already slightly inhibited growth with 40 mM pyruvate and 150 µM tetrachloroethene (PCE) (concentration in the aqueous medium) (Fig. 3.2c). Since lower concentrations were close to the detection limit of gas chromatography, 80 µM 1,2-DBE (concentration in medium) was used for further studies.

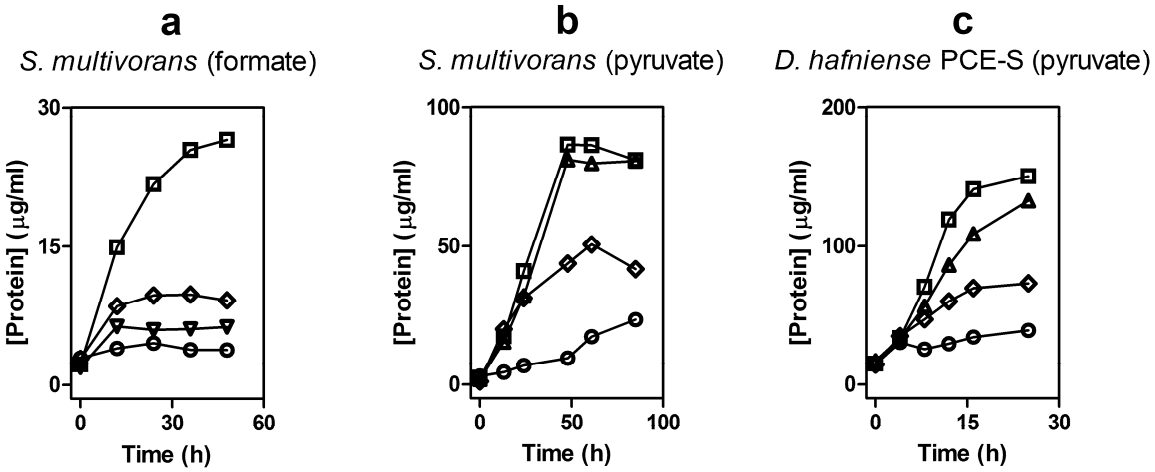

Fig. 3.2 Growth of *S. multivorans* (a, b) and *D. hafniense* PCE-S (c) in media supplemented with 40 mM formate (a) or 40 mM pyruvate (b, c) as electron donors and halogenated ethenes as electron acceptors. 1,2-DBE and/or PCE were applied in a hexadecane phase. Initial concentrations in the aqueous phase were 80 or 150 µM for 1,2-DBE or 150 µM for PCE. Cell densities are given as protein concentrations; different symbols are used for different substrate combinations as follows: ○, formate (a) or pyruvate (b, c) in the absence of halogenated compounds; ◇, formate (a) or pyruvate (b, c) plus 1,2-DBE (80 µM); ▽, formate plus 1,2-DBE (150 µM) (a), △, pyruvate plus PCE plus 1,2-DBE (80 µM) (b, c); □, formate (a) or pyruvate (b, c) plus PCE.

Growth of both *S. multivorans* and *D. hafniense* strain PCE-S with pyruvate was significantly stimulated by 80 µM 1,2-DBE (Fig. 3.2b, c). Again, VB was the only product formed from 1,2-DBE

by *S. multivorans*, whereas *D. hafniense* also formed some ethene (data not shown). In this experiment, 1,2-DBE was quantitatively consumed. The results indicate that both organisms are able to utilize 1,2-DBE as a growth substrate in combination with an appropriate electron donor. To make sure that DBE was not inhibitory for growth under these conditions, a control culture was supplied with both DBE and PCE (Fig. 3.2b, c).

The growth was determined by measuring protein concentrations of disrupted cells as described in the Materials and Methods section. The halogenated ethenes did not influence the protein assay under the experimental conditions applied.

Upon repeated transfer (45 transfers with 10% inoculum) of *S. multivorans* on medium void of halogenated compounds with pyruvate and fumarate as substrates, the PCE dehalogenase (PceA) activity was lost. In such PceA depleted cells the enzyme synthesis could be induced by PCE rather than by chlorinated propenes (John, Rubick *et al.*, 2009). Here, 1,2-DBE (80 µM in the medium) was tested with respect to the ability to induce the PCE dehalogenase synthesis and activity in PceA depleted cells (Fig. 3.3).

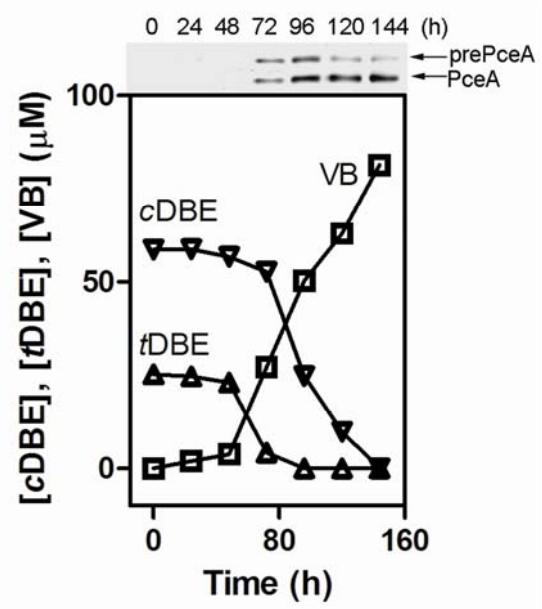

Fig. 3.3 Induction of PceA formation and 1,2-dibromoethene (1,2-DBE) debromination by PceA-depleted *S. multivorans* grown on formate plus 1,2-DBE. PceA was detected by western blot analysis (1 µg protein/lane) performed with PceA-specific antibodies. Sampling times are given above each lane. 1,2-DBE and vinyl bromide (VB) were determined by gas chromatography in the aqueous phase (see Materials and Methods). Δ, *t*DBE; ∇, *c*DBE; □, VB.

The enzyme induction was tested on media containing formate (40 mM) as electron donor and 1,2-dibromoethene (1,2-DBE) (initially 80 µM in the medium) as electron acceptor and was monitored by the debromination of 1,2-DBE measured with gas chromatography and by the detection of PceA via SDS-PAGE using immunoblot with antibodies directed against PceA.

As shown in Fig. 3.3, both the PCE dehalogenase synthesis and debrominating activity as indicated by DBE conversion were induced by 1,2-DBE within 72 hours under the experimental conditions chosen. Both pre- and mature forms of the enzyme were formed. The mature enzyme became the major form after 120 hours. As described by John, Rubick *et al.*(2009), PCE acts as an inducer for the transportation of PceA to periplasm, yielding mature enzyme. The results indicate that 1,2-DBE may also serve as metabolic substrate and inductor for PceA synthesis and export for the organism.

The induction of PceA by 1,2-DBE indicates that this enzyme is responsible for the dehalogenation of brominated ethenes. To verify this assumption, the PCE dehalogenases were purified from both organisms to apparent homogeneity following protocols described earlier (Neumann *et al.*, 1996; Miller *et al.*, 1998) (Fig. 3.4). Upon purification of the enzyme, the ratio of the specific activities with PCE and 1,2-DBE as substrates remained constant (about 2.5:1). From this finding it is concluded that the PCE dehalogenase is the only enzyme in these organisms capable of reductive debromination of 1,2-DBE.

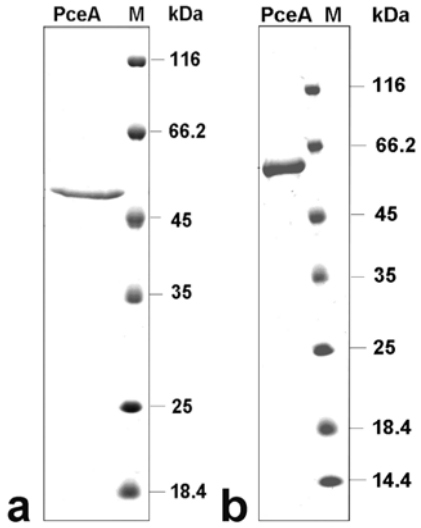

Fig. 3.4 SDS-PAGE of purified tetrachloroethene reductive dehalogenase from *S. multivorans* (a) and *D. hafniense* strain PCE-S (b). Enzyme (2.5 µg) was subjected to electrophoresis in the presence of 0.1% SDS. The gels were stained with Coomassie Blue. PceA, PCE dehalogenase; M, Marker.

3.1.2 Dehalogenation of brominated ethenes by PCE dehalogenases

The dehalogenation of brominated ethenes was tested with purified PCE dehalogenases of *S. multivorans* and *D. hafniense* PCE-S using reduced methyl viologen as artificial electron donor.

Both PCE dehalogenases debrominated 1,2-DBE with apparent K_m values similar to those determined for PCE (Table 3.1; see also Neumann *et al*., 1996; Miller *et al*., 1998). The specific activity of 1,2-DBE dehalogenation was near 40% of that of PCE dehalogenation with PCE dehalogenase of both organisms, as also observed during enzyme purification (see Section 3.1.1). It should be noted that 1,2-DBE was applied as a mixture of *c*DBE and *t*DBE (64/36); therefore, the apparent K_m value should be interpreted with care. From the finding that *t*DBE is converted prior to *c*DBE in whole cells (Fig. 3.1), it may be concluded that the K_m value for *t*DBE might be much lower than for *c*DBE and that the value given in Table 3.1 is a mixture of both values. As described previously for PCE, substrate inhibition was also observed with 1,2-DBE (Table 3.1).

Table 3.1 Kinetic parameters of PCE dehalogenases purified from *S. multivorans* and *D. hafniense* PCE-S and of vitamin B_{12}. For details see Materials and Methods. n. i., no substrate inhibition. PCE, tetrachloroethene; *c*DCE, *cis*-1,2-dichloroethene; 1,2-DBE, 1,2-dibromoethene; TBE, tribromoethene; 2,3-DCP, 2,3-dichloropropene; 2,3-DBP, 2,3-dibromopropene.

substrate	*S. multivorans*			*D. hafniense* PCE-S			Vitamin B_{12}
	Spec. Act. (nkat/mg)[1]	K_m app. (µM)	K_i app. (µM)	Spec. Act. (nkat/mg)[1]	K_m app. (µM)	K_i app. (µM)	Spec. Act. (nkat/mg)[1]
PCE	2333	152	675	103	11	797	0
*c*DCE	0	-	-	0	-	-	0
1,2-DBE	922	162	944	37	36	72	0
TBE	1292	49	n. i.	66	8	n. i.	0
2,3-DCP	392	893	8641	23	319	n. i.	0
2,3-DBP	1360	40	n. i.	82	13	n. i.	26

[1] 1 nkat is defined as 1 nmol halide released or 2 nmol methyl viologen oxidized per sec at 25 °C.

It has been shown that a corrinoid is the cofactor of the PCE dehalogenase (Neumann *et al*., 1995; Miller *et al.*, 1997, 1998; Kräutler *et al.*, 2003). This cofactor also abiotically dehalogenates several halogenated compounds (Neumann *et al.*, 2002). Therefore, as a control we tested vitamin B_{12} as a catalyst for the abiotic reductive debromination (Table 3.1). No reductive debromination of tribromoethene (TBE) or 1,2-DBE was observed with vitamin B_{12} (Table 3.1) or heat-inactivated PCE dehalogenase of both organisms with Ti(III) in the absence and presence of methyl viologen

(data not shown), indicating an enzymatic rather than abiotic conversion of these substrates.

Chlorinated propenes (e. g., 2,3-dichloropropene) have been reported to be substrates for the PCE dehalogenase of *S. multivorans* (Schmitz *et al.*, 2007). Therefore, we tested 2,3-dibromopropene (2,3-DBP) as a substrate for both PCE dehalogenases of *S. multivorans* and *D. hafniense* PCE-S (Table 3.1). With both enzymes, 2,3-DBP was converted at rates, which were about 2.5 times higher than the rates of 2,3-dichloropropene (2,3-DCP) dehalogenation. A very low 2,3-DBP reduction activity was also observed with vitamin B_{12}, indicating a slow abiotic conversion (Table 3.1). In contrast, the reductive dechlorination of 2,3-DCP was only observed with the native enzyme as a catalyst, thus supporting the assumption that bromine substituents are more easily eliminated than chlorine substituents.

Tribromoethene (TBE) appeared to be highly toxic for the organisms even at very low concentrations (data not shown); therefore, growth with this substrate was not determined. However, this compound could serve as a substrate for the PCE dehalogenases of both organisms (Table 3.1). In the dehalogenase assay, Ti(III) citrate was used to reduce methyl viologen (MV) and the resulting reduced MV was oxidized with TBE as electron acceptor.

With crude extract of *S. multivorans*, TBE was converted to *c*DBE, *t*DBE, vinyl bromide (VB) and another compound which was later identified as 1,1-DBE (Fig. 3.5, Fig. 3.6); in a similar experiment with crude extract of *D. hafniense* PCE-S (Fig. 3.6b), also a significant amount of ethene was detected. The same reaction products were observed with the purified PCE dehalogenases and Ti(III) as reductant for methyl viologen (data not shown). The finding that all three isomers of DBE were formed in the enzymatic reaction points to a low stereospecificity of the enzyme when TBE was converted. The formation of all three DBE isomers from TBE is very surprising, since trichloroethene reductive dechlorination by the same enzymes exclusively leads to the formation of *cis*-1,2-dichloroethene.

The identification of 1,1-DBE was as follows. First, a third intermediate peak besides *c*DBE and *t*DBE was observed (retention time: 2.30 min) from gas chromatography with a capillary column (Fig. 3.5a). According to mass spectrometry, this compound contained two bromine substituents, formed vinyl bromide when decomposed, and had the same molecular mass as the two 1,2-DBE isomers (Fig. 3.5b). From these data, it was concluded that this compound was 1,1-dibromoethene.

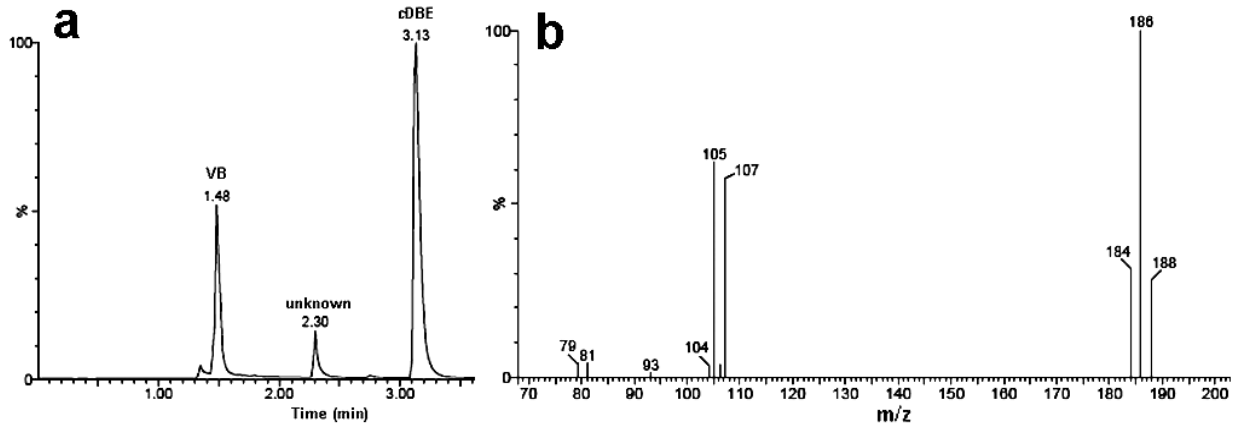

Fig. 3.5 Identification of 1,1-dibromoethene produced during tribromoethene (TBE) dehalogenation with crude extracts from *S. multivorans*. a, Separation of TBE products by gas chromatography. Retention time: *cis*-1,2-DBE (*c*DBE), 3.13 min; unknown, 2.30 min; vinyl bromide (VB), 1.48 min. b. Mass spectrum of the unknown compound (RT = 2.30 min).

In these experiments, the concentrations of *c*DBE detected in the course of the reaction were always by far higher than those of *t*DBE (Fig. 3.6). In whole cells (Fig. 3.1), *c*DBE dehalogenation started only after *t*DBE was almost completely consumed, indicating that *t*DBE is the preferred substrate for the PCE dehalogenase. This might explain why the *t*DBE concentration measured during the enzymatic reaction was always very low due to its fast conversion to VB and why *c*DBE and 1,1-DBE accumulated as intermediates (Fig. 3.6).

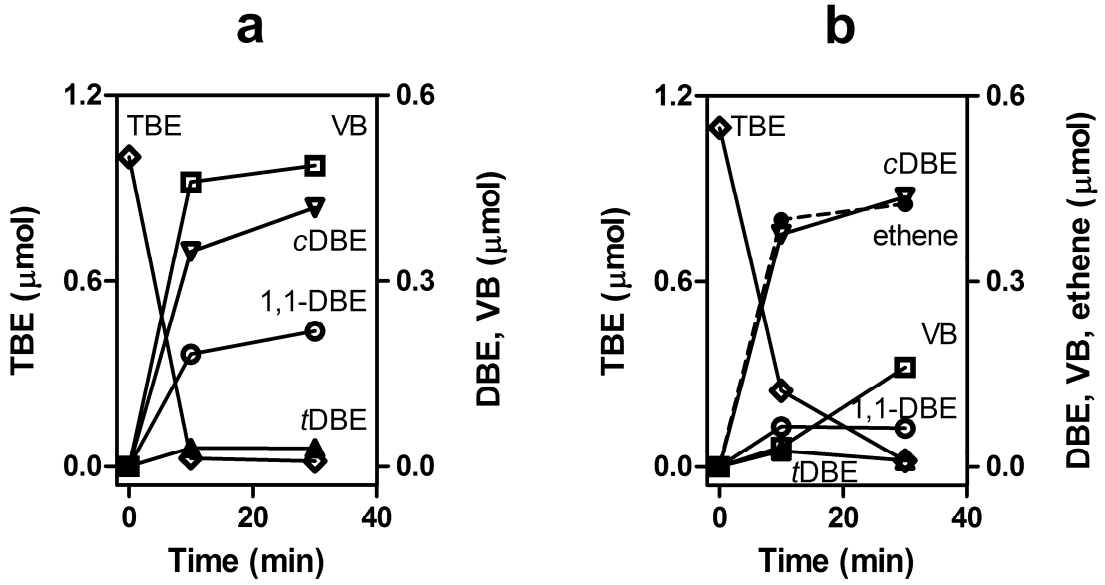

Fig. 3.6 Reductive debromination of tribromoethene by crude extracts of *S. multivorans* (a) and *D. hafniense* PCE-S (b). ◇, TBE; ∆, *t*DBE; ▽, *c*DBE; ○, 1,1-DBE; □, VB; ● with dashed line, ethene (b). Ti(III) was used as reductant for methyl viologen reduction, which served as the electron donor for reductive debromination.

To trace the conversion of TBE via intermediates to the end product(s) in more details and to check whether all three isomers of DBE could be further debrominated to VB, crude extracts of *S. multivorans* with an active PCE dehalogenase and hydrogenase were used. Hydrogen was applied in the gas phase as electron donor for methyl viologen reduction by hydrogenase and hence supplied the reducing equivalents required for TBE debromination. This procedure allowed longer incubation times since reducing equivalents were continuously provided by the hydrogenase. As shown in Fig. 3.7, TBE was converted to VB via *t*DBE, *c*DBE and 1,1-DBE as intermediates, and all isomers of DBE were further debrominated. In case of *D. hafniense* PCE-S, also a low amount of ethene was detected (data not shown).

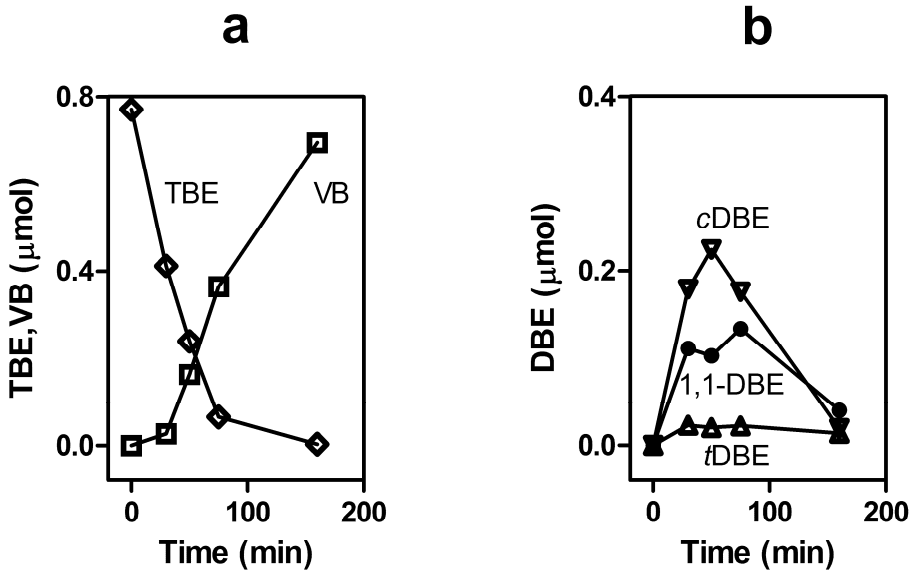

Fig. 3.7 Reductive debromination of tribromoethene (TBE) by crude extracts of *S. multivorans*. a. Conversion of TBE to VB; b. Formation and further conversion of intermediates. ◇, TBE; □, VB; ∆, *t*DBE; ∇, *c*DBE; ●, 1,1-DBE. Hydrogen plus hydrogenase present in the crude extracts was used as reductant for the reduction of methyl viologen. For further details on the experimental conditions, see text and Materials and Methods section.

Ammonium ions were previously shown to stimulate tetrachloroethene and trichloroethene dechlorination by the PCE dehalogenase of *S. multivorans* (Neumann *et al.*, 1996); whereas no stimulation of the dechlorination of chlorinated propenes was observed (Schmitz *et al.*, 2007) (see also Table 3.2). To test the effect of ammonium ions on the reductive dehalogenation of brominated ethenes mediated by the PCE dehalogenase of *S. multivorans*, $(NH_4)_2SO_4$ (4 mM) was added to the enzyme assay with different halogenated substrates. Ammonium ions significantly stimulated the dehalogenation of all halogenated ethenes tested including 1,2-DBE and TBE, whereas no

effect on the dehalogenation of halogenated propenes was observed (Table 3.2). This might indicate different reaction mechanisms of the reductive dehalogenation of halogenated ethenes and propenes as has already been proposed by Schmitz *et al.* (2007), whereas the dehalogenation of chlorinated and brominated ethenes may proceed via a similar reaction mechanism.

Table 3.2 Effect of $(NH_4)_2SO_4$ (4 mM) on the dehalogenation of different halogenated alkenes with the PCE dehalogenase purified from *S. multivorans*. PCE, tetrachloroethene; 1,2-DBE, 1,2-dibromoethene; TCE, trichloroethene; TBE, tribromoethene; 2,3-DCP, 2,3-dichloropropene; 2,3-DBP, 2,3-dibromopropene.

Substrate	Specific activity (nkat/mg)		Factor
	$-NH_4^+$	$+NH_4^+$	
PCE	1029	2333	2.27
1,2-DBE	698	922	1.32
TCE	932	2239	2.40
TBE	547	1292	2.36
2,3-DCP	408	391	0.96
2,3-DBP	1508	1360	0.90

Previous studies on the reductive dechlorination of chlorinated propenes by the PCE dehalogenase of *S. multivorans* showed that this reaction probably occurs via a radical mechanism (Schmitz *et al.*, 2007). Here, we performed similar experiments with 1,2-DBE or 2,3-DBP as substrates and compared the results with the kinetics of the dechlorination of PCE and 2,3-DCP (shown for the PCE dehalogenase of *S. multivorans* in Fig. 3.8).

Limited concentrations of the halogenated electron acceptors as compared to those of methyl viologen (MV) were applied. This was done to ensure that the halogenated substrate was completely consumed before the methyl viologen was re-reduced. Using this procedure, further enzymatic oxidation of MV after re-reduction with Ti(III) citrate was avoided. With halogenated propenes as substrates, MV could not be completely re-reduced, indicating the formation of MV adducts with radical reaction intermediates (Fig. 3.8; see also Schmitz *et al.*, 2007). 1,2-DBE and TBE (not shown) debromination and PCE dechlorination did not lead to the loss of MV. Essentially the same results were obtained with purified PCE dehalogenase of *D. hafniense* PCE-S (data not shown).

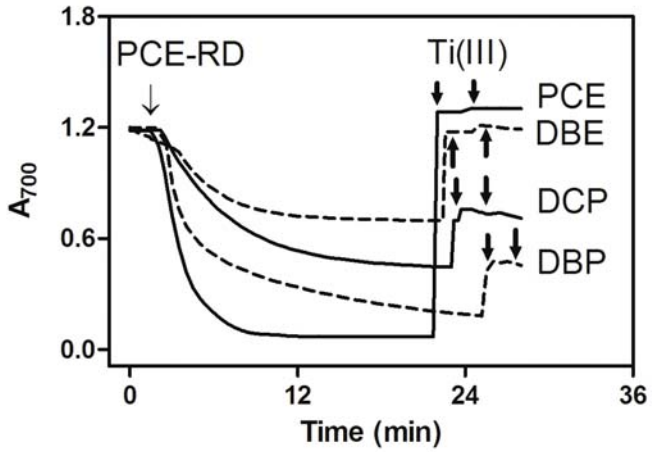

Fig. 3.8 Reaction kinetics of the enzymatic conversion of limiting concentrations of tetrachloroethene (PCE), 1,2-dibromoethene (1,2-DBE), 2,3-dichloropropene (2,3-DCP), and 2,3-dibromopropene (2,3-DBP) with methyl viologen (MV) as electron donor. 0.5 mM MV was initially completely reduced with Ti(III) citrate. After addition of halogenated substrate, the reaction was started with enzyme (~3 µg purified PCE dehalogenase for *S. multivorans*; thin arrow). When the reaction rate was near 0 indicating a complete conversion of the halogenated substrate, Ti(III) citrate was added repeatedly (thick arrows) until a maximum of absorption (A_{700}) was reached. PCE-RD, PCE dehalogenase; Ti(III), Ti(III) citrate.

3.2 Reactions of fungal peroxidases involving halogen anions

3.2.1 Purification and characterization of halogenating peroxidases from *Bjerkandera adusta* strain Ud1

As reported by Verhagen *et al.* (1996), *Bjerkandera adusta*, *Hypholoma fasciculare*, *Mycena epipterygia* and *Hypholoma sublateritium* belong to the most potent producers of halogenated metabolites. Synthesis of halogenated compounds by these fungi has been intensively studied (Spinnler *et al*., 1994; Verhagen *et al*., 1998a), however, no halogenating enzyme has been reported so far. These fungi were therefore chosen for investigation of the production of halogenating enzymes.

Production of halogenating peroxidases

Grown in liquid high nitrogen content medium supplemented with 1 mM chloride or bromide, all the fungal strains were tested for growth yield and halogenating activity using the monochlorodi-

medone (MCD) assay (Hager *et al*., 1966). All cultures (1 *Hypholoma fasciculare* strain, 3 *Hypholoma sublateritium* strains, 4 *Bjerkandera adusta* strains, 1 *Mycena epipterygia* strain) grew well in this medium, with a minimal growth yield of 1.75 g/l. Using the monochlorodimedone assay, significant halogenating activity was only detected in *Bjerkanda adusta* cultures (data not shown).

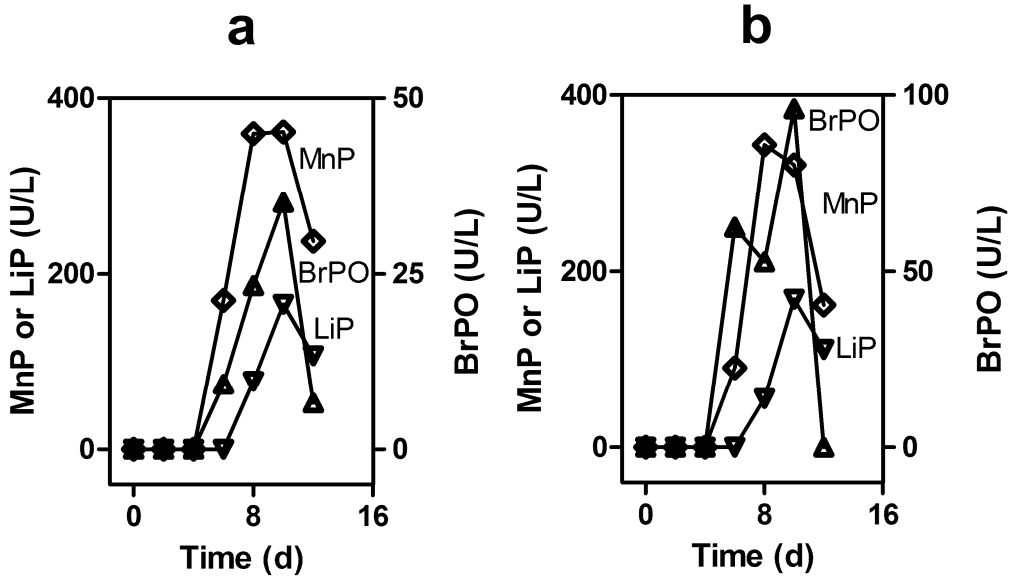

Fig. 3.9 Kinetics of peroxidase production by *Bjerkandera adusta* strain Ud1 in high nitrogen content culture supplemented with 1 mM NaCl (a) or NaBr (b). One unit is defined as 1 µmol MCD consumed in dependence of bromide (BrPO) or 1µmol veratryl alcohol oxidized (LiP) or 1µmol Mn(III)-malonate formed (MnP) per minute. △, BrPO, brominating peroxidase activity measured with MCD assay, with unspecific MCD oxidation activity subtracted; ▽, LiP, lignin peroxidase activity; ◇, MnP, manganese peroxidase activity. For details on the enzyme assays, see Materials and Methods section.

Among all the fungal strains tested, in all *Bjerkandera adusta* strains with halogenating activity, ligninolytic activities such as manganese peroxidase and lignin peroxidase activities were also detected (Fig. 3.9); whereas in fungi lacking halogenating activity no lignin degrading enzyme activity was measured under the conditions tested (data not shown).

To check whether this brominating activity is a bio-catalytic activity or an abiotic reaction, a portion of active culture fluid was heat-inactivated (95ºC, 20 min) and then subjected to the assay. No significant activity was detected in heat-inactivated sample with either monochlorodimedone (MCD) assay or phenol red assay. These observations led to the conclusion that at least one native enzyme capable of bromination was present.

Bjerkandera adusta strain Ud1 was chosen for further investigation of halogenating enzymes due to its relatively high halogenating activity in culture fluid. In both NaCl and NaBr supplemented

media, day 10 seemed to be the best time for harvesting and subsequent purification of halogenating peroxidases (Fig. 3.9). In both cultures, the brominating peroxidase activities and the lignolytic enzyme activities showed similar kinetics.

Purification and characterization of halogenating peroxidases from B. adusta strain Ud1

10-day-old liquid cultures grown in high nitrogen content medium supplemented with 1 mM NaCl or NaBr and 6 week-old wood cultures of *B. adusta* strain Ud1 were used for purification of halogenating peroxidases.

In the following, results obtained with halogenating peroxidases purified from a 10-day-old NaCl supplemented liquid culture are described as an example. The monochlorodimedone assay was used to screen all the fractions eluted from the columns for brominating activity, using a control assay without halide ions. After the first anion exchange column, Q-Sepharose, two activity peaks were found with the MCD assay (Fig. 3.10a). Since they were able to brominate MCD, they were named as brominating peroxidase (BrPO)-1 and BrPO-2. It should be noted that an unspecific MCD oxidation occurred in the assays (not exceeding about 50% of the total enzyme activity). The brominating activities given here are the values corrected for this unspecific peroxidase activity.

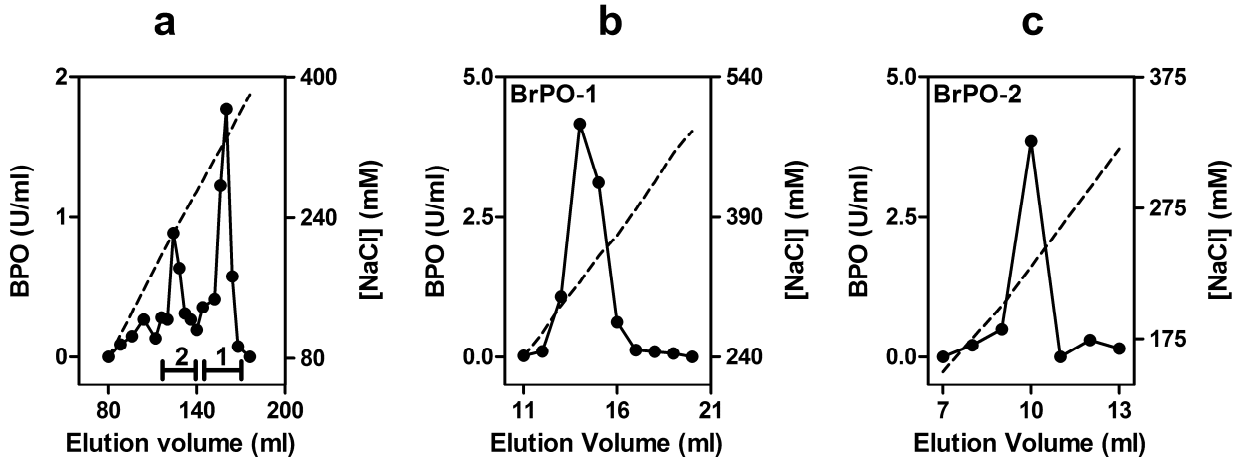

Fig. 3.10 Activity profiles of brominating peroxidases (BrPO) during purification using different chromatography columns. The culture used for purification was 10 days old grown in high nitrogen content medium supplemented with 1 mM NaCl. a. Q-Sepharose column for the separation of the culture fluid; b. Mono Q column chromatography with the BrPO-1 fractions eluted from the Q-Sepharose column; c. Mono Q column with the BrPO-2 fractions eluted from the Q-Sepharose. The bars in a indicate the BrPO-1 and BrPO-2 fractions that were pooled for next column. ●, brominating activity; dashed line, NaCl concentration in the elution buffer.

Each brominating peroxidase activity peak was pooled and further purified with a second anion exchange chromatography of finer resolution using a Mono Q column. On this column, only one activity peak was found (Fig. 3.10b, c). At this stage, the enzymes (BrPO-1 and BrPO-2) were purified to apparent homogeneity as shown by SDS-PAGE (Fig. 3.11a).

The same results were obtained with NaBr supplemented liquid cultures; whereas in wood cultures, a single brominating activity peak was eluted from the Q-Sepharose. Later (see below, Fig. 3.12) it was confirmed that this enzyme was different from BrPO-1 and BrPO-2; hence, it was designated BrPO-3. This enzyme was purified to apparent homogeneity (Fig. 3.11b) by chromatography on Q-Sepharose followed by a hydrophobic interaction chromatography using a Phenyl-Superose column as described in Materials and Methods section.

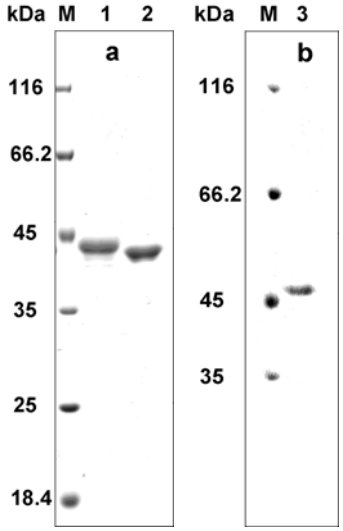

Fig. 3.11 SDS-PAGE of purified brominating peroxidases from *B. adusta* Ud1 grown in high nitrogen content medium with NaCl (a) or wood medium (b). The protein (1.0 µg per lane) was subjected to electrophoresis in the presence of 0.1% SDS. The gels were stained with Coomassie Blue. M, Marker; 1, BrPO-1; 2, BrPO-2; 3, BrPO-3. The BrPOs purified from NaBr-supplemented high nitrogen content medium showed the same size as shown in a (not shown). For experimental details, see Materials and Methods section.

To check whether the different brominating peroxidases (BrPOs) were identical enzymes, the 45-kDa or 43-kDa bands on a denaturing gel of the BrPOs were excised, and their tryptic digests were analyzed by MALDI-TOF fingerprinting as well as Tandem Mass Spectrometry in combination with an online analysis of the resulting data using the National Center for Biotechnology Information (NCBI) database. The MALDI-TOF finger printing indicated that the BrPO-1 and BrPO-2 purified from NaCl-supplemented liquid culture were the same proteins as the BrPO-1 and BrPO-2 from

NaBr-supplemented culture, respectively, whereas the BrPO-3 from wood culture was a different protein (Fig. 3.12).

From BrPO-1, four tryptic peptides (vacpdgvntatnaaccalfavrddiqqnlfdggecgeevheslr, gtlfpgtsg-nqgevesplageir, tacewqsfvnnqpr, lsilghdltqmidcsdvipvppstavr) with unambiguous sequence tags gave matches with 29.4% coverage to the 37-kDa **R**emazol Brilliant Blue R ***B****jerkandera* **P**eroxi-dase a (RBPa) precursor from *Bjerkandera* spp. B33/3 (Accession number: AAO47909.1) and with 29.5% coverage to the 45-kDa versatile peroxidase of *Bjerkandera adusta* strain MZKI G-84 (Accession number: AAY89586.1), whereas no match was found with BrPO-2 and BrPO-3.

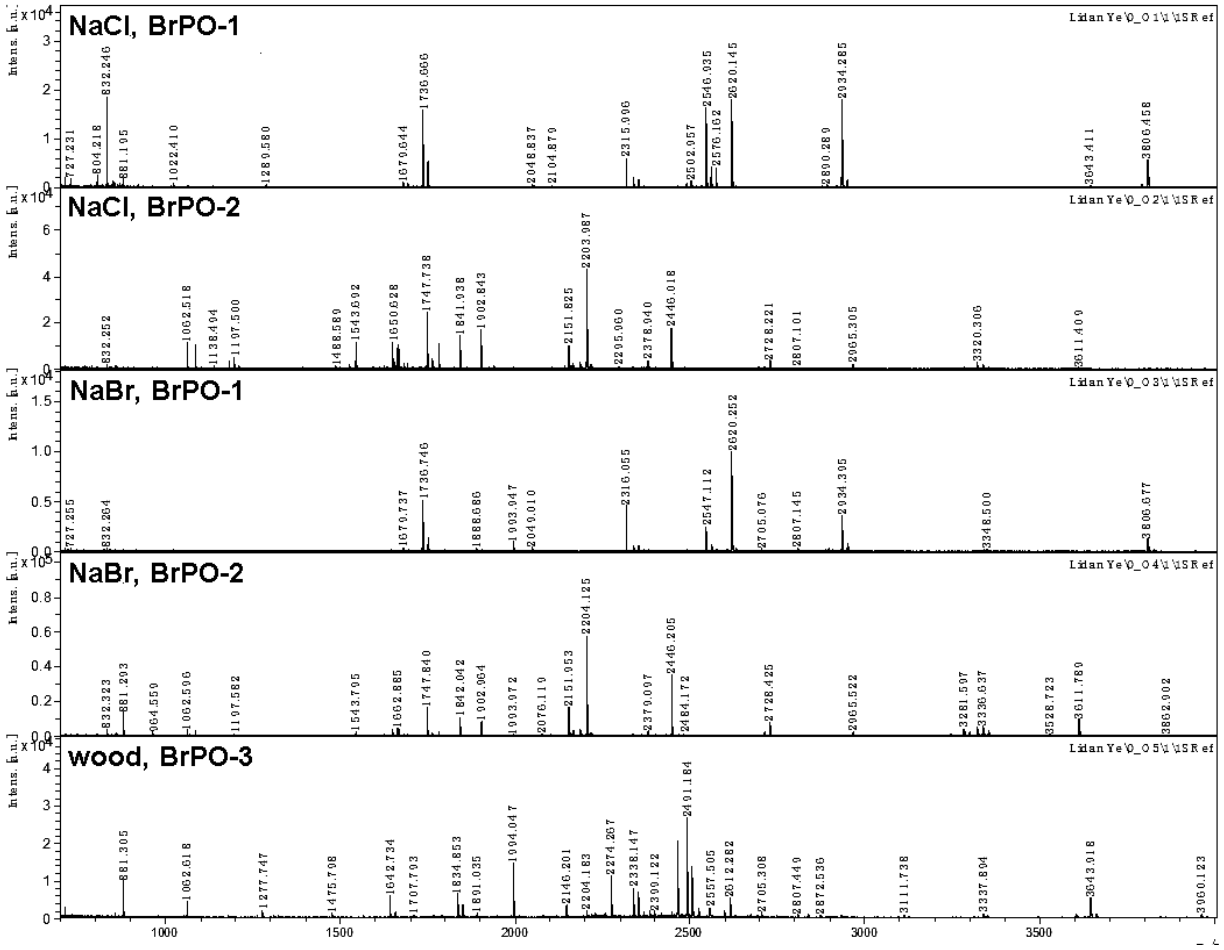

Fig. 3.12 MALDI-TOF finger printing of brominating peroxidases (BrPOs) purified from *B. adusta* strain Ud1 cultures grown in different media.

The absorption spectra of each BrPO purified from *B. adusta* strain Ud1 in their oxidized or reduced form were recorded. These spectra looked similar; an example is shown for BrPO-2 in Fig. 3.13. For all BrPOs, a typical UV/VIS-spectrum of a heme protein was exhibited, with decreased

heme absorption upon oxidation and a shift of the heme soret band from 405 nm to 435 nm upon reduction (see also Fahnenschmidt *et al.*, 2001).

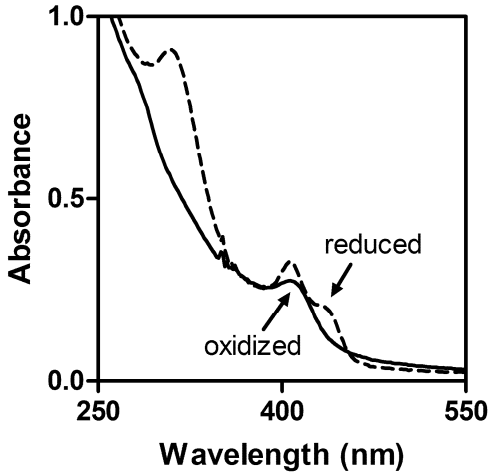

Fig. 3.13 Absorption spectra of purified BrPO-2. The enzyme was reduced with 1 mM sodium dithionite or oxidized with 1 mM hydrogen peroxide.

N-terminal amino acid sequences of these BrPOs were determined and found to be highly conserved among the peroxidases from *Bjerkandera* species (Table 3.3). The first 25 amino acids of BrPO-1 and BrPO-3 are identical to all known versatile peroxidases from *Bjerkandera* spp., whereas the first 25 amino acids of BrPO-2 are almost identical to the known lignin peroxidases of *Bjerkandera* spp., with the only difference in the 22nd amino acid (C instead of R). All BrPOs share part of the amino acid sequence with the versatile peroxidases from *Pleurotus ostreatus* (Table 3.3). In comparison to the sequences of the chloroperoxidase of *Caldariomyces fumago*, the halogenating peroxidases of *Phanerochaete chrysosporium* or the halogenating peroxygenase of *Agrocybe aegerita*, the similarity was very low (Table 3.3).

Substrate spectrum and pH optima of the brominating enzymes

Since the brominating peroxidases (BrPOs) obtained from NaCl-grown cultures and NaBr-grown cultures were identical, only the BrPO-1 and BrPO-2 from NaCl-grown culture and BrPO-3 from wood-grown culture were used for further studies.

Besides the bromination in the monochlorodimedone assay, the oxidation of bromide and iodide was also measured for each BrPO by the formation of the tribromide (Br_3^-) and triiodide (I_3^-) complexes, respectively (data not shown; see also Libby *et al.*, 1982; Brown and Hager, 1967).

Table 3.3 N-terminal sequences of halogenating peroxidases from *Bjerkandera adusta* strain Ud1 and comparison with known fungal peroxidases. Mismatches with *B. adusta* VPs are underlined. X in the sequence usually represents a cysteine. BrPO, brominating peroxidase; VP, versatile peroxidase; LiP, lignin peroxidase; HPO, haloperoxidase.

Strain	Enzyme (Type)	N-terminal sequence	Reference
B. adusta Ud1	BrPO-1 (BrPO)	VACPDGVNTATNAACCALFAVRDDI	This work
B. adusta Ud1	BrPO-2 (BrPO)	VACPDGRHTAINAACCNLFTVCDDI	This work
B.adusta Ud1	BrPO-3 (BrPO)	VACPDGVNTATNAACCALFAVRDDI	This work
Bjerkandera sp. B33/3	RBP (VP)	VAXPDGVNTA	Moreira *et al.,* 2006
Bjerkandera sp. BOS55	MnP-LiP hybrid (VP)	VACPDGVNTATNAACCALFAVRDDI	Mester and Field, 1998
Bjerkandera sp. BOS55	BOS1 (VP)	VAXPDGVNTATNAAXXXLFAVRDDI	Palma *et al.,* 2000
	BOS2 (VP)	VAXPDGVNTATNAAXXALFAVRDDI	
B. adusta DSM11310	MnP1 (VP)	VAXPDGVNTATNAAXXALFAVRDDI	Heinfling *et al.,* 1998
B. adusta UAMH8258	MnP1 (VP)	VAXPDGVNTATNAAXXALFA	Wang *et al.,* 2002
Bjerkandera sp. BOS55	LiP-2 (LiP)	VACPDGRHTAINAACCNLFTVRDDI	ten Have *et al.*, 1998
Bjerkandera sp. BOS55	LiP-5 (LiP)	VACPDGRHTAINAACCNLFTVRDDI	ten Have *et al.*, 1998
Pleurotus ostreatus	MnP2 (VP)	ATCADGRTTA-NAACCVLFPILDDI	Giardina *et al.,* 2000
P. eryngii	MnPL1 (VP)	VTCATGQTTANEAXXALFPI	Camarero *et al.,* 1999
	MnPL2 (VP)	VACPDGVNTATNAACCALFAVRDDI	
P. eryngii	PS1 (VP)	VAXPDGVNTATNAAXXXLFAVRDDI	Ruiz-Duenas *et al.,* 1999
	PS3 (VP)	VTCADGNTV	
Agrocybe aegerita	AaP II a (HPO)	EPGKPPGPP-ESSA	Ullrich *et al.,* 2004
	AaP II b (HPO)	EPGKP-GPPEES--	
Phanerochaete chrysosporium	LiP H8 (LiP)	ATCSNGKTVGDASS	PDB: 1B85A
Caldariomyces fumago	CPO (HPO)	EPGSGIGYPYDNNT	NCBI: CAC16733

Brominating activity of each BrPO was also measured using the phenol red assay, in which phenol red was brominated to bromophenol blue, giving rise to a characteristic absorption spectrum with a maximum at 592 nm. In addition, phenol bromination was measured in the presence of bromide, BrPO and hydrogen peroxide, with 2-bromophenol as major product. A minor amount of 4-bromophenol was also detected. The conversion was monitored by HPLC and the reaction products were identified with internal standards (Fig. 3.14).

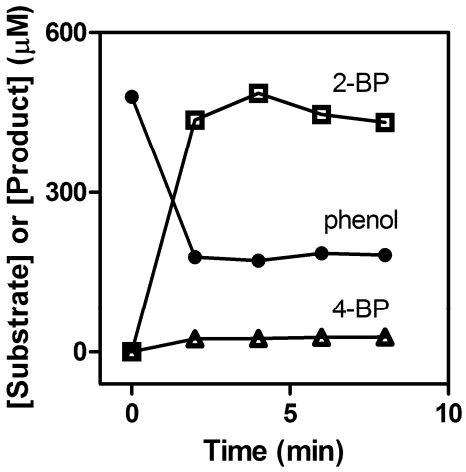

Fig. 3.14 Bromination of phenol by BPOs. The reaction mixture (1 ml) contained 20 mM phosphate buffer (pH 3.0), 10 mM NaBr, 500 µM phenol, 5 µg BrPO-3 and 1 mM H_2O_2 (reaction time 10 min). ●, phenol, □, 2-bromophenol (2-BP), △, 4-bromophenol (4-BP).

Enzymatic activities of all three BrPOs were tested with substrates for haloperoxidase (MCD and halide ions), manganese peroxidase (Mn^{2+}), lignin peroxidase (veratryl alcohol) as well as laccase (ABTS). Specific activities were calculated for each substrate and listed in Table 3.4. All three BrPOs exhibited both lignin peroxidase and manganese peroxidase activities. Hence, these BrPOs obviously belong to the family of versatile peroxidases (VPs) and were renamed as VP-1, VP-2 and VP-3.

In the monochlorodimedone assay, all four halides were tested and except chloride, addition of all other halide ions significantly enhanced the consumption of monochlorodimedone (MCD) (Table 3.4). The fluoride-dependent MCD consumption is very surprising, as it indicated a seemingly peroxidase-catalyzed fluorinating reaction (described in more details in Section 3.2.2).

Table 3.4 Specific activities and substrate spectra of *B. adusta* strain Ud1 VPs and CPO from *Caldariomyces fumago*. VP, versatile peroxidase; CPO, chloroperoxidase; LiP, lignin peroxidase activity; MnP, manganese peroxidase activity; MCD, monochlorodimedone. -, no reaction detected.

Enzyme	Organism	LiP (U/mg)	MnP (U/mg)	MCD oxidation (U/mg)	MCD halogenation[1] (U/mg)			
					F^-	Cl^-	Br^-	I^-
VP-1	*B. adusta*	10.4	51.1	14.2	29.3	-	6.4	70.7
VP-2	*B. adusta*	24.0	0.9	2.4	3.4	-	4.4	87.3
VP-3	*B. adusta*	26.8	0.3	1.3	2.9	-	19.9	19.6
CPO	*C. fumago*	-	-	-	-	2087	3526	77.6

[1]MCD halogenation activity was corrected with MCD oxidation activity, if there was any.

The pH optima of the different peroxidase reactions were determined for all three *B. adusta* strain Ud1 versatile peroxidases (VPs) (Table 3.5). When monochlorodimedone together with different halides were supplied as substrates, a pH optimum between 2.0 and 3.0 was observed, which is comparable to previously reported pH optima of the halogenating activities of *Caldariomyces fumago* chloroperoxidase (CPO) (Shaw and Hager, 1961) and ***A**grocybe **a**egerita* **P**eroxidase (AaP) (Ullrich *et al.*, 2004). For the lignin peroxidase and manganese peroxidase activities, using veratryl alcohol or Mn(II) as substrate, the pH optima for the VPs were determined to be 3.0 - 3.5 and 4 - 4.5, respectively. These results were similar to those obtained with lignin peroxidases (ten Have *et al.*, 1998) and manganese peroxidases (Périé *et al.*, 1996) of various fungal species.

Table 3.5 pH optima of the *B. adusta* Ud1 versatile peroxidases and other fungal peroxidases with different substrates. -, no reaction detected; n.d., not determined. VP, versatile peroxidase; LiP, lignin peroxidase; AaP, *Agrocybe aegerita* peroxidase; CPO, chloroperoxidase.

Enzyme	Organism	pH optimum						Reference
		Mn(II)	VA	MCD	MCD + Cl^-	MCD + Br^-	MCD + F^-	
VP-1	*B. adusta*	4.5	3.0	3.0	-	3.0	3.0	This work
VP-2	*B. adusta*	4.0	3.0	3.0	-	2.0	2.0	This work
VP-3	*B. adusta*	n.d.	3.5	3.5	-	2.0 – 3.0	2.0 – 3.0	This work
LiP-2	*B.* BOS5	-	2.5	n.d.	n.d.	n.d.	n.d.	ten Have *et al.*, 1998
LiP-5	*B.* BOS5	-	3.0	n.d.	n.d.	n.d.	n.d.	
MnPII	*Dichomitus squalens*	5.0	-	n.d.	n.d.	n.d.	n.d.	Périé *et al.*, 1996
AaP	*Agrocybe aegerita*	-	7.0	n.d.	3.0	3.0	n.d.	Ullrich *et al.*, 2004
CPO	*Caldariomyces fumago*	-	-	-	3.0	3.0	-	Shaw and Hager, 1961

Enzyme kinetics of the versatile peroxidases purified from Bjerkandera adusta strain Ud1

With Mn(II) and veratryl alcohol as substrates, the apparent K_m values of VP-1 through VP-3 were measured at pH 4.5 and 3.0, respectively, and compared to those of the previously described versatile peroxidases from *Bjerkandera* species (Table 3.6). According to the K_m values, the versatile peroxidases purified are obviously isoenzymes different from the published **R**emazol Brilliant Blue R ***B**jerkandera* **P**eroxidase (RBP) and the versatile peroxidase "MnP-LiP hybrid".

Table 3.6 Apparent K_m values of versatile peroxidases (VPs) purified from *Bjerkandera* strains with MnP or LiP substrates. RBP, Remazol Brilliant Blue R *Bjerkandera* peroxidase; MnP, manganese peroxidase; LiP, lignin peroxidase.

Enzyme	Organism	Substrate	pH	App. K_m (μM)	Reference
VP-1	*B. adusta* Ud1	Mn(II)	4.5	165	This work
		Veratryl alcohol	3.0	806	
VP-2	*B. adusta* Ud1	Mn(II)	4.5	2886	This work
		Veratryl alcohol	3.0	451	
VP-3	*B. adusta* Ud1	Mn(II)	4.5	11	This work
		Veratryl alcohol	3.0	167	
MnP-LiP hybrid	*Bjerkandera spp.* BOS55	Mn(II)	4.5	51	Mester and Field, 1998
		Veratryl alcohol	3.0	116	
RBP	*Bjerkandera spp.* B33/3	Mn(II)	5.0	86	Moreira *et al.,* 2006
		Veratryl alcohol	3.0	1500	

The kinetic constants of halide (bromide or fluoride)-dependent MCD consumption catalyzed by VPs were investigated at pH 3.0. The apparent K_m and K_i values calculated for halide ions as well as for H_2O_2 are shown in Table 3.7. It seems that in the presence of fluoride the inhibitory effect caused by high concentrations of hydrogen peroxide was relieved as indicated by the higher apparent K_i values determined for hydrogen peroxide when 20 mM fluoride was present. The presence of fluoride also increased the apparent K_m of the enzymes for H_2O_2 (Table 3.7).

Table 3.7 Apparent K_m and K_i values of versatile peroxidases (VPs) with Br^- or F^- or H_2O_2 as substrates. The reaction mixtures contained 100 mM citrate/phosphate buffer, 0.7 μg VP-1 or 2.0 μg VP-2 or 1.0 μg VP-3, 2.0 mM H_2O_2 and different concentrations of the halides, or 20 mM NaBr or NaF and different concentrations of H_2O_2.

Enzyme	0.1 mM MCD		0.1 mM MCD + 20 mM Br^-			0.1 mM MCD + 20 mM F^-		
	App. K_m [H_2O_2] (μM)	App. K_i [H_2O_2] (μM)	App. K_m [Br^-] (mM)	App. K_m [H_2O_2] (μM)	App. K_i [H_2O_2] (μM)	App. K_m [F^-] (mM)	App. K_m [H_2O_2] (μM)	App. K_i [H_2O_2] (μM)
VP-1	6.9	1074	13.1	36.6	391.9	9.1	208.2	12019
VP-2	50.9	205.4	19.7	694.6	182.4	19.7	366.7	14508
VP-3	39.1	196.3	18.3	727.9	94.7	14.2	179.1	1588

In contrast to the effect of fluoride, the presence of bromide did not relieve inhibition caused by high concentrations of H_2O_2 (no significant change in apparent K_i values), but rather increased the apparent K_m values for H_2O_2.

Heme bleaching of VP-3 in the presence of hydrogen peroxide plus bromide or fluoride was

investigated using a halide ion-free assay as control. The presence of 40 µM bromide led to fast heme bleaching when the hydrogen peroxide concentration reached 40 µM (Fig. 3.15b), whereas no significant difference was observed with the same concentration of fluoride compared to the control (Fig. 3.15a, c). It has been reported previously that no heme bleaching was observed with iodide, probably due to the lower redox potential of I_2/HOI and the instability of HOI under acidic conditions (Sheng and Gold, 1997). Since HOF is unstable at room temperature, the formation of HOF as a short-living intermediate could not be excluded so far.

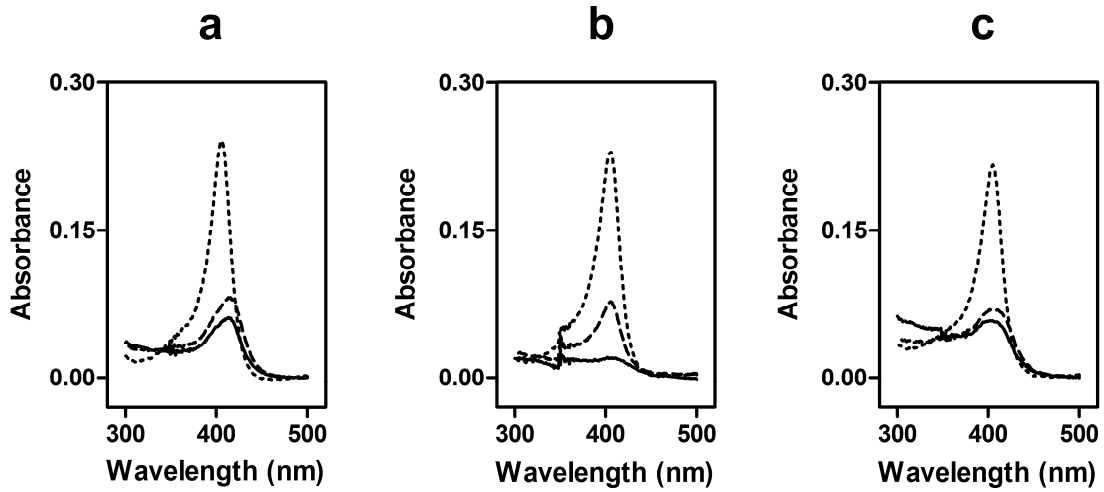

Fig. 3.15 Bleaching of the heme of VP-3 in the presence of hydrogen peroxide plus bromide or fluoride. a. Halide ion-free control; b. 40 µM bromide; c. 40 µM fluoride. The reaction mixture contained 1 µM VP-3 in 100 mM citrate/phosphate buffer, pH 2.8; hydrogen peroxide was added as 20 µM aliquots, starting with 0 µM and ending with 40 µM. Dotted line: 0 µM H_2O_2; dashed line: 20 µM H_2O_2; continuous line: 40 µM H_2O_2.

VP-3 was also studied for the influence of other halides on monochlorodimedone (MCD) halogenation (Table 3.8). An unspecific MCD oxidation was observed in the absence of halide anions and the addition of 20 mM chloride did not stimulate this reaction. Addition of 5 mM chloride or fluoride to the assay with 20 mM bromide caused a 24% and a 44% inhibition of the bromination, respectively. When the 5 mM sodium chloride was added to an assay containing 20 mM fluoride, no significant effect was observed.

All these findings indicated the fluoride-dependent MCD consumption might proceed via a different mechanism from the brominating reaction and therefore needed further investigation.

Table 3.8 Conversion of monochlorodimedone by VP-3. Reaction mixtures (1 ml) consisted of 100 mM citrate/phosphate buffer pH 2.8, containing MCD (0.1 mM), NaF or NaCl or NaBr (20 mM), H_2O_2 (1.0 mM), and enzyme (1.2 µg VP-3)**.** MCD halogenation or oxidation was monitored as a decrease in the absorbance at 280 nm.

Assay	Rate of MCD halogenation and/or oxidation (U/mg)
MCD + H_2O_2	5.81
MCD + Cl^- + H_2O_2	5.56
MCD + Br^- + H_2O_2	28.66
MCD + Br^- + Cl^- (5 mM) + H_2O_2	16.15
MCD + Br^- + F^- (5 mM) + H_2O_2	21.72
MCD + F^- + H_2O_2	8.60
MCD + F^- + Cl^- (5 mM) + H_2O_2	8.53
MCD + F^- + Br^- (5 mM) + H_2O_2	11.35

3.2.2 Characterization of fluoride-dependent peroxidase reactions mediated by manganese peroxidase of *Bjerkandera adusta* strain Ud1

The versatile peroxidases described in Section 3.2.1, a lignin peroxidase and a manganese peroxidase of *B. adusta* strain Ud1 as well as MnPs from other fungi was tested for their ability to brominate monochlorodimedone (MCD) and for the reaction of fluoride in this assay (Table 3.9).

Table 3.9 Fluoride-dependent monochlorodimedone (MCD) consumption activity of manganese peroxidases (MnPs) and lignin peroxidase (LiP) from different fungal species. One unit of MnP is defined as 1 µmol Mn(III)-malonate formed per minute. One unit of F^--dependent MCD consumption is defined as 1 µmol MCD consumed in dependence on F^- per minute.

Species	Enzyme	MnP (U/ml)	F^--dependent MCD consumption (U/ml)
Clitocybula dusenii b11	MnP	4.2	0.3
Phlebia frowardii b19	MnP	14.3	1.4
Pleurotus eryngii	MnPII	59.2	2.7
B. adusta Ud1	MnP-1	183.2	7.4
B. adusta Ud1	Crude LiP	0	0

All MnPs tested were found to mediate the fluoride-dependent consumption of MCD (Table 3.9) rather than bromination of MCD (not shown), whereas the LiP of *B. adusta* strain Ud1 did not convert MCD in dependence on fluoride (Table 3.9). Hence, it is feasible that the fluoride-dependent MCD conversion is a general feature of manganese peroxidases. The MnP-1 of *B. adusta* strain Ud1 was chosen for further studies on the reaction with fluoride.

Purification of MnP-1 from Bjerkandera adusta strain Ud1

Mn(III)-malonate formation was measured photometrically to determine the MnP activity as described in the Materials and Methods section. The single MnP activity peak eluted from Q-Sepharose (not shown) was applied to a Mono Q column after pooling and dialyzing, which resulted in the elution of two activity peaks (Fig. 3.16a).

Peak I had majorly only MnP activity, whereas peak II had both Mn(II) and veratryl alcohol oxidation activities, i. e., versatile peroxidase activity. Peak I was pooled, dialyzed and separated with a second Mono Q column, which led again to the elution of two activity peaks (Fig. 3.16b); the two enzymes were designated MnP-1 and MnP-2.

After an additional hydrophobic interaction chromatography (Phenyl-Superose), MnP-1 was eluted as one single activity peak (Fig. 3.16c) and appeared to be homogenous according to SDS-PAGE with an apparent molecular mass of about 43 kDa (Fig. 3.17).

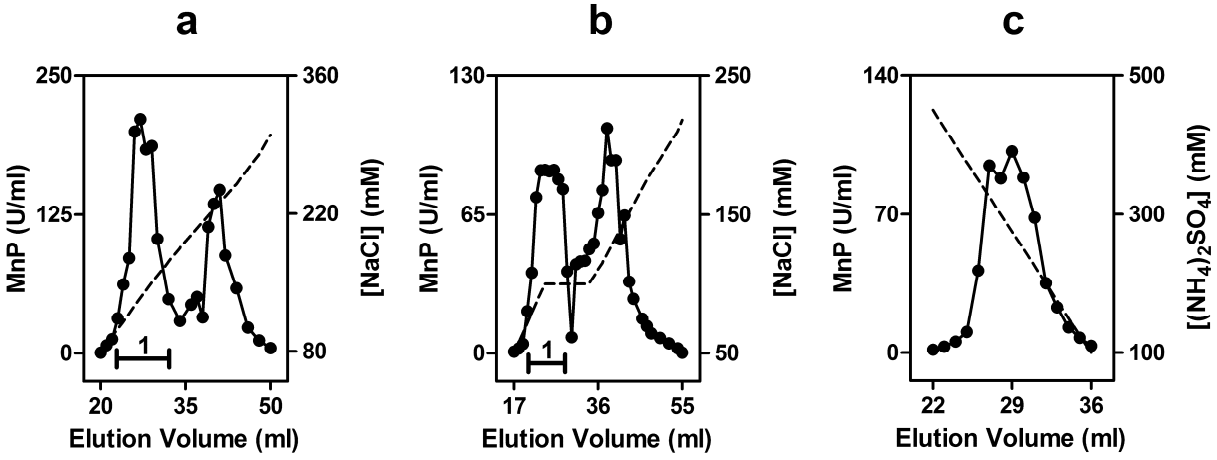

Fig. 3.16 Elution profile of MnP-1 from *Bjerkandera adusta* strain Ud1 culture fluids. a. Mono Q column after Q-Sepharose purification; b. second Mono Q column using a different NaCl gradient with samples pooled from the first activity peak in a; c. Phenyl-Superose column with samples pooled from the first activity peak in b. Dash line, NaCl or $(NH_4)_2SO_4$ (c) concentration. Bars indicate the fractions pooled for the next columns.

Substrate spectrum of MnP-1 mediated fluoride-dependent reactions

In the absence of Mn(II), the monochlorodimedone (MCD) consumption mediated by *B. adusta* strain Ud1 MnP-1 was tested in the presence and absence of fluoride or bromide ions (Fig. 3.18a). MCD conversion was only observed in the presence of fluoride.

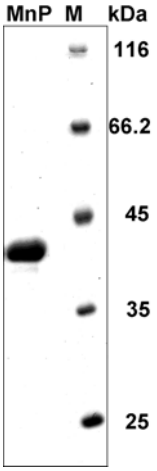

Fig. 3.17 SDS-PAGE of purified MnP-1 from *B. adusta* strain Ud1. Enzyme (~2 µg) was subjected to electrophoresis in the presence of 0.1% SDS. The gels were stained with Coomassie Blue.

Both fluoride anions and hydrogen peroxide were essentially required for this reaction (Fig. 3.18b). At first glance, MCD consumption in the presence of fluoride indicated a fluoroperoxidase reaction. This is against the well-accepted knowledge about fluorination and thus needed further investigation.

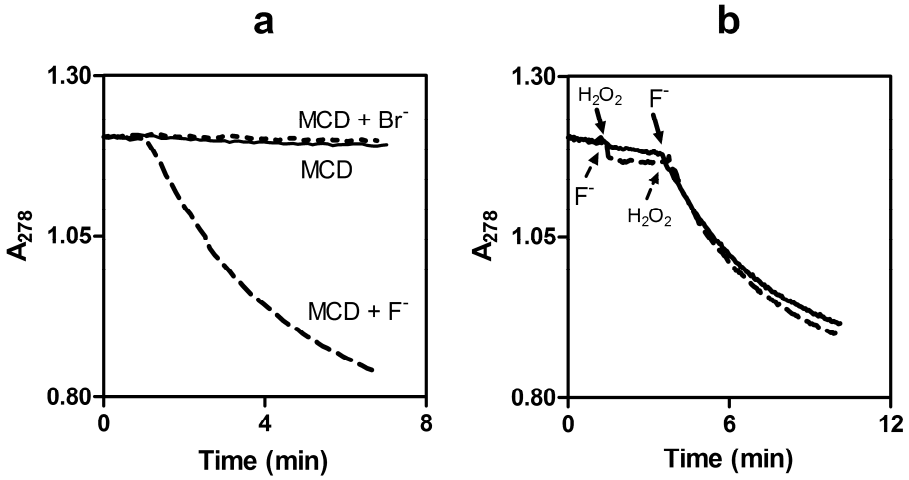

Fig. 3.18 Fluoride-dependent monochlorodimedone (MCD) consumption catalyzed by MnP-1 of *Bjerkandera adusta* strain Ud1. a. MCD consumption in the absence or presence of different halide anions. b. Test for the requirement of the reaction for both fluoride anions and H_2O_2. The reaction mixture contained 1.5 µg MnP-1, 0.1 mM MCD, 20 mM NaBr or NaF, where indicated, and 2.0 mM H_2O_2 in 100 mM citrate/phosphate buffer, pH 2.8. The reaction in **a** was started by addition of H_2O_2. In **b**, dashed line: fluoride was added in prior to H_2O_2; continuous line: H_2O_2 was added in prior to fluoride.

MnP-1 was also tested with respect to the effect of fluoride in other peroxidases assays using 2,6-dimethoxyphenol, guaiacol, ABTS, 1,2,3,5- tetramethoxybenzene or veratryl alcohol as substrates. All the fluoride-dependent reactions were performed in the absence of Mn(II). Except 1,2,3,5-tetramethoxybenzene and veratryl alcohol, which have a high redox potential of near 1.1 V

(Kersten *et al.*, 1990) and 1.4 V (Fawer *et al.*, 1991; Branchi *et al.*, 2005) under the conditions applied, respectively, all other substrates were converted by MnP-1 in the presence of F^- and H_2O_2 (Table 3.10).

In the assay with ABTS, an increase in absorption at 420 nm was measured indicating the oxidation of this substrate to $ABTS^+$. 2,6-dimethoxyphenol appeared to be converted to a dimer and guaiacol was probably oxidized to a tetramer as indicated by the absorption increase at 469 nm or 470 nm, respectively. The absorption increase in the assays indicated that these substrates were oxidized by MnP-1 in the presence of H_2O_2 and fluoride. The reactions were strictly dependent on the presence of fluoride in addition to H_2O_2.

Table 3.10 Substrate spectrum and pH optimum of *B. adusta* strain Ud1 MnP-1. For the determination of specific activities, 100 mM citrate/phosphate buffer (pH 2.8) was used for fluoride-dependent reactions, whereas 50 mM malonate buffer (pH 4.5) was used for Mn(II)-dependent reactions. 2,6-DMP, 2,6-dimethoxyphenol; MCD, monochlorodimedone; VA, veratryl alcohol.

Substrate	Ion present	$[H_2O_2]$ (mM)	Specific activity (U/mg)	pH optimum
ABTS (0.3 mM)	20 mM F^-	1.0	29.7	3.5
2,6-DMP (1.0 mM)	20 mM F^-	1.0	21.1	3.0
MCD (0.1 mM)	20 mM F^-	1.0	19.9	3.0 - 3.5
guaiacol (1.0 mM)	20 mM F^-	1.0	2.0	3.5
1,2,3,5-TMB	20 mM F^-	1.0	0	-
VA (4.0 mM)	20 mM F^-	1.0	0	-
Mn(II) (0.5 mM)	0.5 mM Mn(II)	0.2	259.2	4.5 -5.5

The pH dependence of the MnP-catalyzed fluoride-dependent reactions including MCD consumption and oxidation of various substrates is also shown in Table 3.10. With all the substrates tested, the optimal pH for fluoride-dependent substrate conversion was always 3.0 - 3.5. In comparison, the oxidation of Mn(II) to Mn(III) had a pH optimum around 5.0 (Table 3.10). The acidic pH optima of the fluoride-dependent reactions again point to a different reaction mechanism compared to the classical manganese peroxidase reaction with Mn(II) as substrate.

High concentrations of fluoride (20 mM) were required for a reasonable velocity of this reaction, whereas low concentrations of Mn(II) as the regular substrate were sufficient to obtain high activities (Table 3.10). Therefore, it is feasible that the activity observed with fluoride might be due to an contamination of NaF with Mn(II), which would explain the unusual MnP-1 mediated reaction with

fluoride in the peroxidase assays.

To exclude false positive reactions caused by a potential Mn(II) impurity, NaF of the highest purity available (99.99% pure; <0.1 ppm Mn(II); Sigma-Aldrich, Steinheim, Germany) was used in the assays. In addition, special care was taken for cleansing of the glassware used for the assay. To estimate the concentration of Mn(II) as a potential contaminant, we measured MnP-1 catalyzed 2,6-dimethoxyphenol (DMP) oxidation with H_2O_2 and F^- or Mn(II). About the same activity was measured with 20 mM NaF and 150 µM Mn(II) (Fig. 3.19a). From this result, it could be concluded that if the fluoride-dependent activity was really caused by the Mn(II) impurity in NaF, there should be as much as 150 µM Mn(II) in 20 mM NaF, which corresponded to about 10,000 ppm Mn(II) in NaF. Due to the purity of the chemicals used (<0.1 ppm Mn(II)), it appears unlikely that the fluoride-dependent oxidation reactions are caused by a contamination with Mn(II).

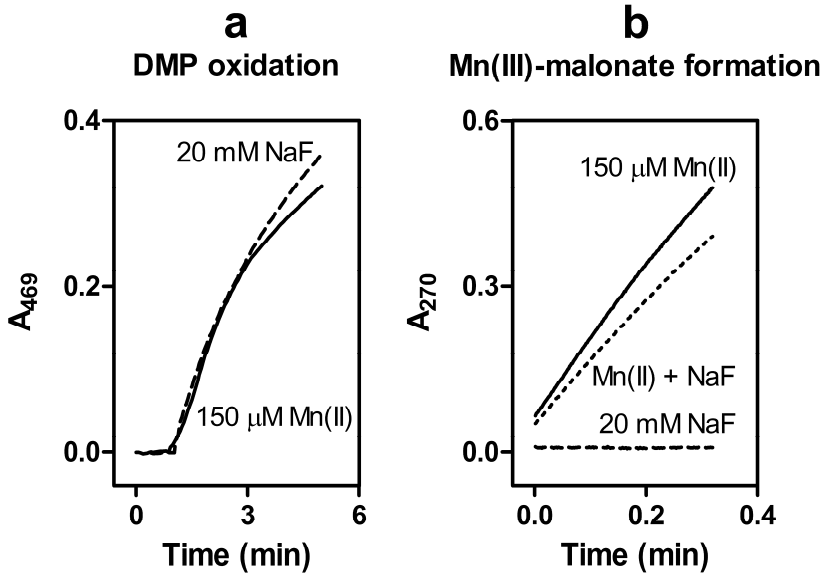

Fig. 3.19 Oxidation of 2,6-dimethoxyphenol (DMP) and formation of Mn(III)-malonate by MnP-1 of *B. adusta* strain Ud1 in the presence of 20 mM NaF and/or 150 µM $MnCl_2$. a. Oxidation of dimethoxyphenol in the presence of Mn(II) or fluoride. The reaction was conducted in 100 mM citrate/phosphate buffer (pH 4.0), with 1.5 µg MnP-1, 0.2 mM DMP and 0.1 mM H_2O_2 in the presence of 20 mM NaF or 150 µM $MnCl_2$. b. Manganese(III)-malonate formation in the presence of Mn(II) and/or fluoride. The reaction was conducted in 50 mM malonate buffer (pH 4.5) with 0.2 mM H_2O_2 in the presence of 20 mM NaF and/or 150 µM $MnCl_2$.

Furthermore, if there was a significant amount of Mn(II) present as impurity in NaF, the formation of Mn(III)-chelate complex should be observed with NaF. Therefore, 20 mM NaF were applied in the MnP assay in the presence of 50 mM malonate and the formation of the Mn(III)-malonate

complex was measured photometrically (Fig. 3.19b). No formation of this complex was observed (dashed line in Fig. 3.19b), which indicated no significant amount of Mn(II) should be present in NaF. As controls, 150 µM Mn(II) in the absence and presence of 20 mM NaF was applied. A high activity with Mn(II) was measured; the activity was only slightly inhibited by NaF (Fig. 3.19b). From these experiments, a contamination of NaF with Mn(II) can be excluded. Hence, the fluoride-dependent oxidation seems to represent a novel type of MnP mediated reaction. The reaction was further investigated with respect to the formation of intermediates and details on the substrate conversion.

Haloperoxidases mediate halogenation by formation of hypohalous acid, which then unspecifically reacts with the substrates. Hypohalous acids are known to inactivate peroxidases in the absence of substrates, which could be halogenated and thus serve as scavengers (Sheng and Gold, 1997; Hasan *et al.*, 2006). Therefore, if hypofluorous acid was formed by MnP-1 in the presence of H_2O_2 and F^-, it should lead to the inactivation of the enzyme in the absence of an organic substrate. The inactivation was tested under different assay conditions given in Fig. 3.20a.

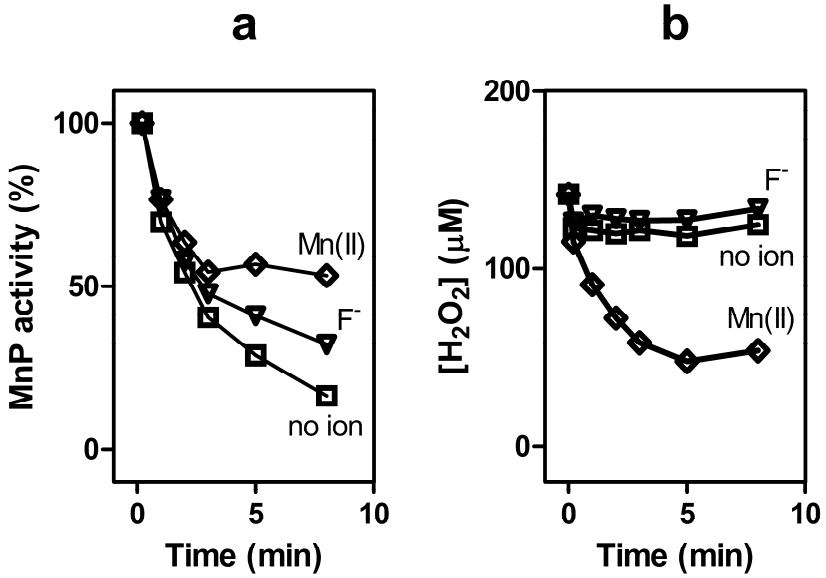

Fig. 3.20 Inactivation of *B. adusta* strain Ud1 MnP-1 at pH 4.0. a. Inactivation of MnP under different conditions, monitored by Mn(III)-malonate formation rate; b. Hydrogen peroxide concentration during MnP inactivation. Reaction mixture contained about 3 µg MnP-1, 0.1 mM H_2O_2 and 20 mM NaF or 0.5 mM $MnCl_2$ where stated in 100 mM citrate/phosphate buffer, pH 4.0. □, no fluoride; ▽, 20 mM fluoride; ◊, 0.5 mM Mn(II).

The enzyme was incubated in the assay containing H_2O_2 in the presence or absence of NaF or Mn(II). Samples were taken at the times indicated and the Mn(II) oxidation was measured in the

regular MnP assay. For details on the experimental conditions, see Materials and Methods section. In the presence of H_2O_2 together with fluoride, the enzyme was not inactivated faster than in the presence of H_2O_2 alone; in fact, fluoride appeared to slightly relieve the inactivation exerted by H_2O_2. This result indicated that MnP-1 did not mediate the formation of hypofluorous acid from fluoride anions in the presence of hydrogen peroxide (Fig. 3.20a). This observation points to a fluoride-dependent reaction completely different from the haloperoxidase reactions described so far.

Since no hypofluorous acid seems to be formed by MnP-1 in the assay, it was tested if H_2O_2 was consumed in such an assay containing NaF, or Mn(II) as a control. Hydrogen peroxide consumption was only observed when Mn(II) was present (Fig. 3.20b). The decrease of the H_2O_2 concentration in the presence of Mn(II) indicated the oxidation of Mn(II) as a substrate. In the presence of fluoride and in the absence of an organic substrate, hydrogen peroxide was not consumed, indicating oxidation of fluoride did not occur. It is therefore feasible that fluoride does not serve as a substrate for MnP-1.

Identification of the products of the fluoride-dependent reactions

Since hypofluorous acid was obviously not formed in the hydrogen peroxide- and fluoride-dependent conversions mediated by MnP-1 of *B. adusta* strain Ud1, fluorination of the organic substrates might not occur. To gain more information on the fluoride-dependent reactions, the reaction products of 2,6-dimethoxyphenol conversion in the presence of fluoride were subjected to LC-MS analysis and compared with those formed in the presence of manganese.

The same reaction products were observed with LC-MS for MnP-1 mediated 2,6-dimethoxyphenol (DMP) conversion with fluoride or with manganese, as indicated by their identical LC spectra and mass spectra (Fig. 3.21a, b). The products had a molecular mass of 305 (RT=17.5 min) and 291 (RT=17.3 min), respectively. High Resolution Mass Spectrometry analysis of these reaction products suggested a molecular formula of $C_{16}H_{17}O_6$ and $C_{15}H_{15}O_6$, respectively (Fig. 3.22). Therefore the major product should be a dimer of 2,6-dimethoxyphenol, whereas the minor product with a molecular mass of 291 was a dimer where a methyl group was missing. This finding was not surprising since *O*-demethylation activity has been described for fungi (Ibrahim *et al.*, 2003).

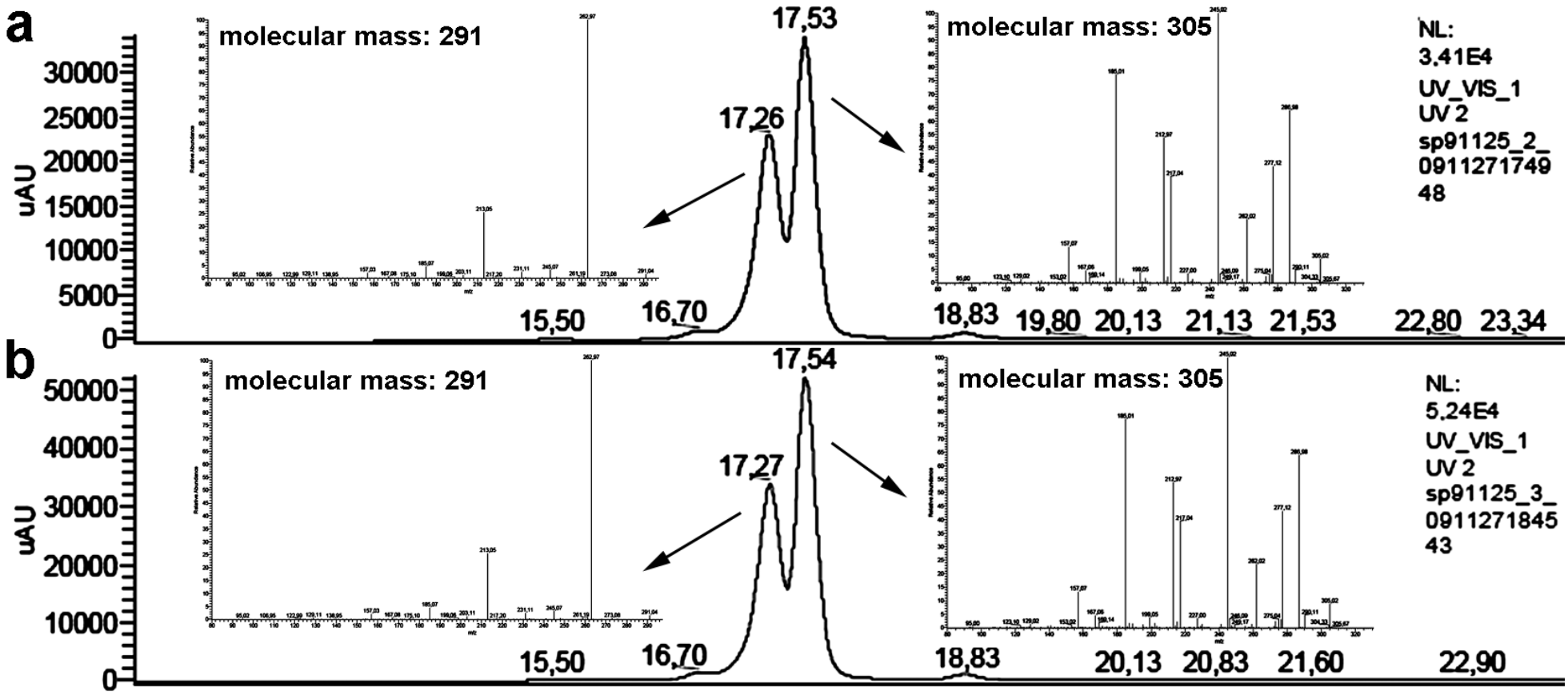

Fig. 3.21 LC-MS analysis of reaction products of MnP-1 mediated 2,6-dimethoxyphenol conversion in the presence of fluoride (a) or manganese (b). The mass spectra of the products are shown as insets. The reaction was in 100 mM citrate/phosphate buffer (pH 4.0), with 0.2 mM DMP, 3 µg MnP-1, 0.1 mM H_2O_2, and 20 mM NaF or 150 µM $MnCl_2$.

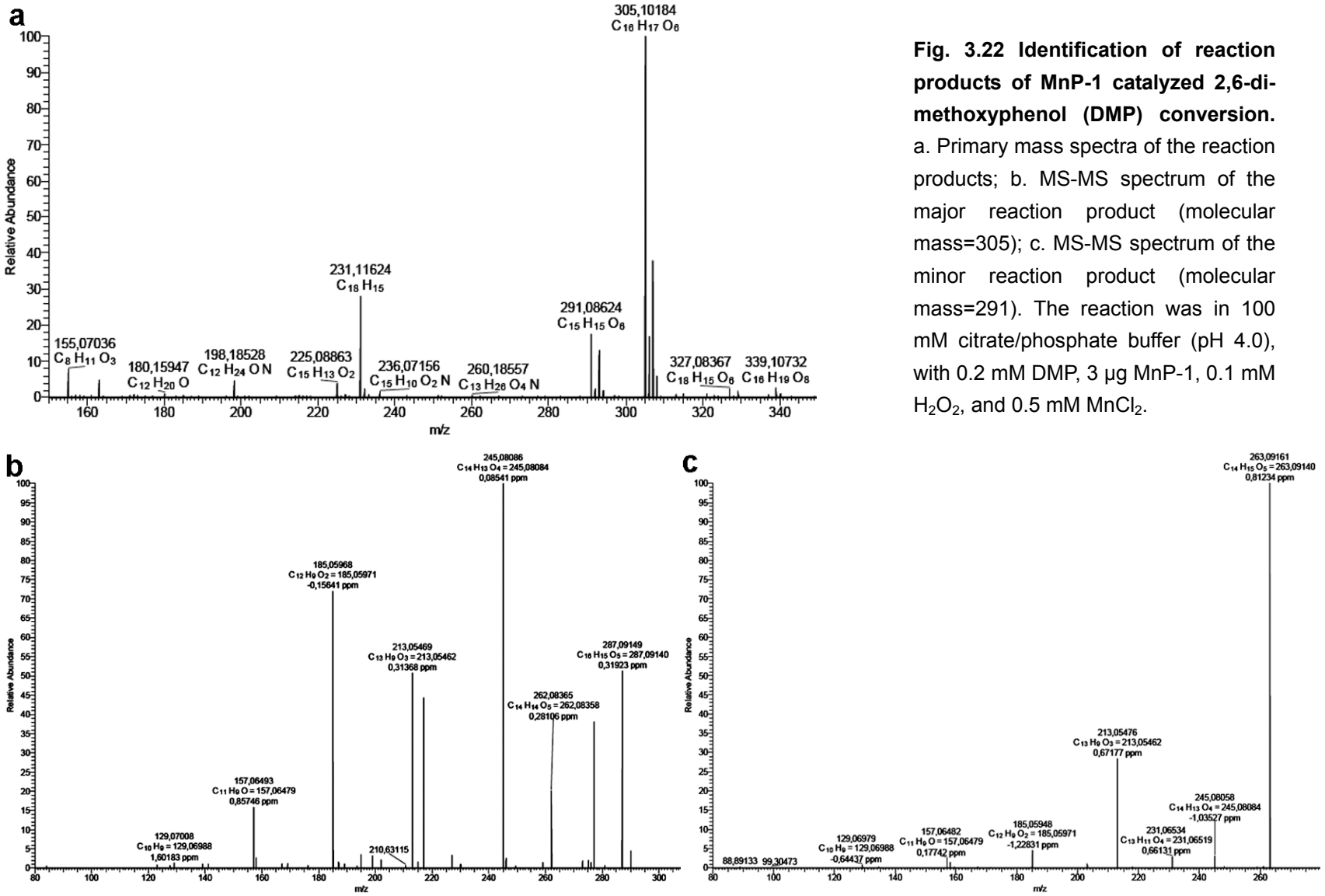

Fig. 3.22 Identification of reaction products of MnP-1 catalyzed 2,6-di-methoxyphenol (DMP) conversion. a. Primary mass spectra of the reaction products; b. MS-MS spectrum of the major reaction product (molecular mass=305); c. MS-MS spectrum of the minor reaction product (molecular mass=291). The reaction was in 100 mM citrate/phosphate buffer (pH 4.0), with 0.2 mM DMP, 3 µg MnP-1, 0.1 mM H_2O_2, and 0.5 mM $MnCl_2$.

The conversion of DMP to the products was observed in a stoichiometric ratio of 2:1 in the presence of either fluoride or manganese (Fig. 3.23), assuming they have the same extinction coefficient at 469 nm as 3,3',5,5'-tetramethoxy-4,4'-diphenoquinone (ε_{469}=27.5 $mM^{-1}cm^{-1}$; Stajic *et al.*, 2004). This stoichiometric ratio supported that dimers were formed during the conversion of DMP by MnP-1 either with fluoride or manganese. MnP-1 was rapidly inactivated during these reactions (not shown), which might be explained by the radical formation during DMP oxidation (Wariishi *et al.*, 1992). These findings led to the conclusion that in DMP oxidation fluoride was not a real substrate for MnP-1 but rather an agent that altered the substrate spectrum of the enzyme, enabling it to oxidize 2,6-dimethoxyphenol directly in the absence of manganese.

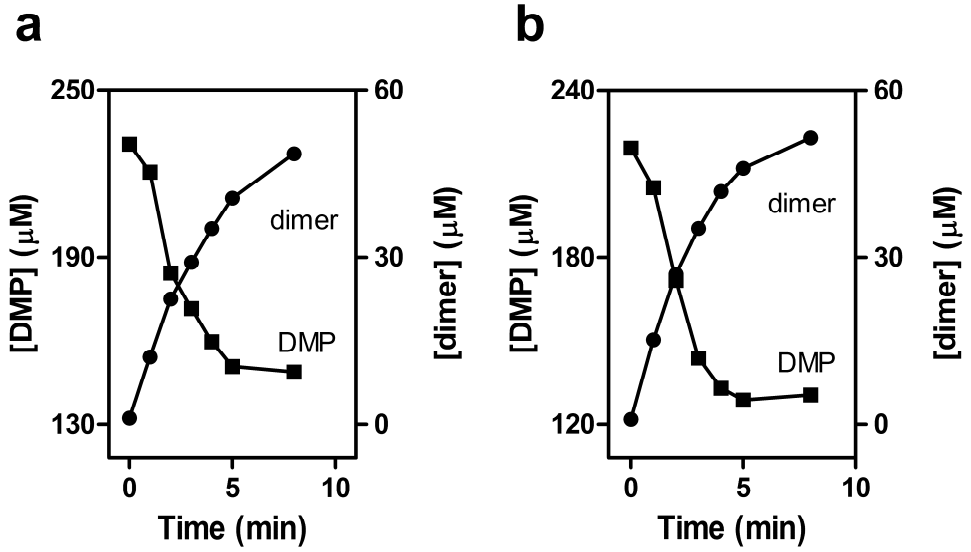

Fig. 3.23 Stoichiometry of MnP-1 mediated 2,6-dimethoxyphenol oxidation in the presence of fluoride (a) or manganese (b). The reaction was in 100 mM citrate/phosphate buffer (pH 4.0), with 0.2 mM DMP, 3 µg MnP-1, 0.1 mM H_2O_2, and 150 µM $MnCl_2$ or 20 mM NaF.

The monochlorodimedone (MCD) and hydrogen peroxide consumption was tested in the MCD assay with either fluoride or Mn(II) in the presence of MnP-1. The MCD concentration was determined by HPLC. MCD and hydrogen peroxide were consumed in a stoichiometry of near 1:1 in the presence of 20 mM NaF (Fig. 3.24a). No product could be detected with either HPLC or LC-MS or GC-MS (data not shown). The HPLC analysis was performed by measuring the absoprtion at 210 and 278 nm. Since aromatic or quinoid compounds as well as organic acids should be detected at least at 210 nm, it is feasible that the MCD conversion led to the formation of smaller molecules that did not absorb at these wavelengths. Hence, fluorination of MCD in the reaction could be excluded and oxidation of MCD was instead suggested.

MCD was not converted in the absence of fluoride and in the presence of Mn(II), although the consumption of H_2O_2 indicated the formation of Mn(III) (Fig. 3.24b). This again indicates the fluoride-dependent reaction mediated by MnP-1 might have occurred via a different mechanism from that of the Mn(II)-dependent reaction.

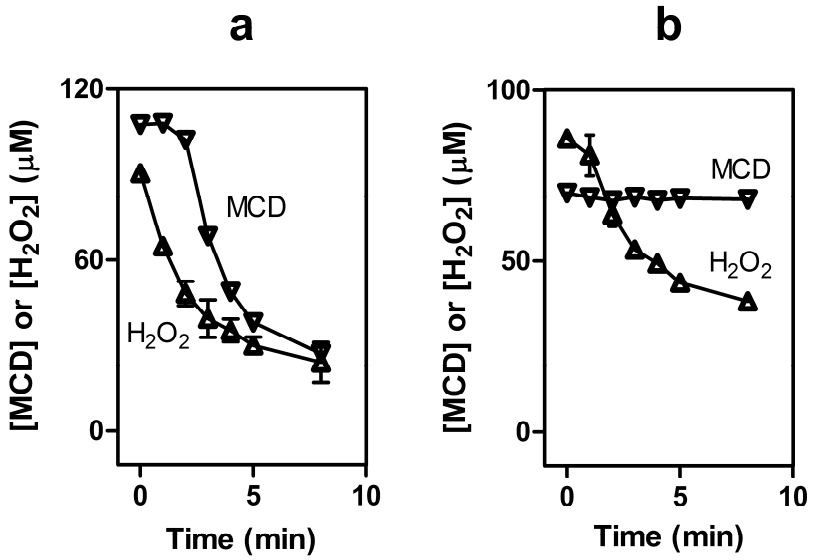

Fig. 3.24 Kinetics of monochlorodimedone (MCD) and H_2O_2 consumption in the presence of fluoride (a) or Mn(II) (b). The reaction was conducted in 100 mM citrate/phosphate buffer (pH 4.0) with 0.1 mM MCD and 0.1 mM H_2O_2 in the presence of 20 mM NaF or 150 μM $MnCl_2$. Values shown here were the mean of triplicates.

Kinetic parameters of MnP-1 catalyzed fluoride-dependent reactions

Monochlorodimedone (MCD) and 2,6-dimethoxyphenol (DMP) were applied as substrates for study on the enzyme kinetics of the fluoride-dependent reactions mediated by MnP-1. The apparent kinetic constants are shown in Table 3.11.

Table 3.11 Apparent K_m and K_i values of substrates in the reactions catalyzed by MnP-1 in the presence or absence of fluoride. Fluoride-dependent reactions were carried out in 100 mM citrate/phosphate buffer (pH 2.8) containing 0.6 μg MnP-1, whereas Mn(II) oxidation was in 50 mM malonate buffer (pH 4.5). MCD, monochlorodimedone; DMP, 2,6-dimethoxyphenol.

Ion present	App. K_m (mM)[1]	App. K_m H_2O_2 (mM)[2]	App. K_i H_2O_2 (mM)[2]
F^- (20 mM)	0.29 (MCD)	0.20	3.0
F^- (20 mM)	0.71 (DMP)	1.60	1.0
-	0.10 (Mn(II))	0.02	1.7

[1]The reactions contained 1 mM H_2O_2 in the presence or absence of 20 mM NaF.

[2]The reactions contained 0.1 mM MCD, 1 mM DMP or 0.5 mM Mn(II) in the presence or absence of 20 mM NaF.

The K_m value for hydrogen peroxide in Mn(II) oxidation was lower (< 10%) than that in the fluoride-dependent reactions. For all reactions, high concentration of hydrogen peroxide had an inhibitory effect on the reaction, with a K_i value ranging from 1.0 mM to 3.0 mM (Table 3.11).

For the fluoride-dependent reactions, high concentrations of NaF (20 mM), especially compared to the concentration of e. g. MCD (0.1 mM), had to be applied. If fluoride serves as a substrate and is converted stoichiometrically, it would not be possible to detect fluoride consumption. Therefore, it is not surprising that with a fluoride electrode a decrease of the fluoride concentration could not be observed. In addition, no fluorinated reaction product was formed in the assays as indicated by HPLC, LC-MS as well as GC-MS. Even though fluoride obviously was not a substrate for MnP-1, the dependence of the reaction rate on the fluoride concentration obeyed Michaelis-Menten kinetics (Fig. 3.25).

The rate of monochlorodimedone or 2,6-dimethoxyphenol consumption increased with increasing fluoride concentration with about 90% of the maximal velocity at near 35 or 66 mM NaF, respectively. High fluoride concentrations (5.6 or 9.6 mM) were required to obtain half-maximal reaction velocity.

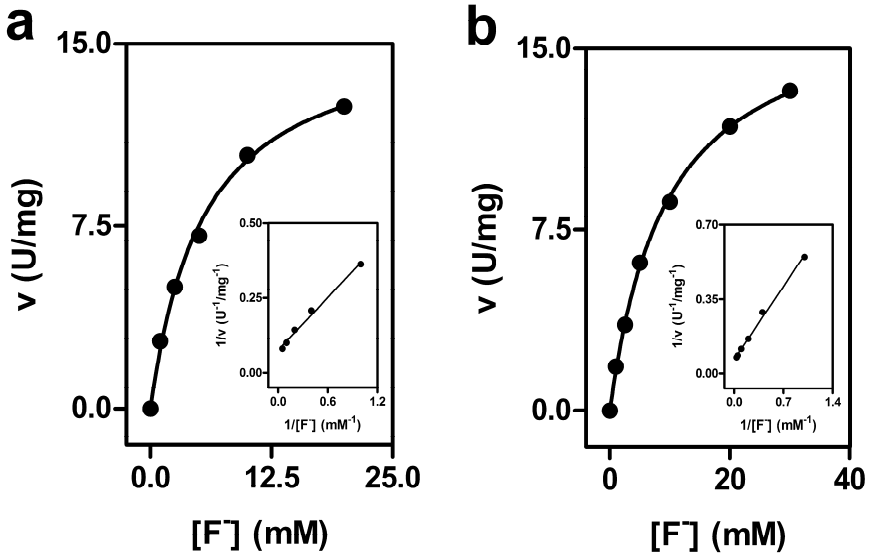

Fig. 3.25 Reaction rate of monochlorodimedone (MCD) (a) or 2,6-dimethoxyphenol (DMP) (b) consumption in dependence on the fluoride concentration. The Lineweaver-Burk plots are shown as insets. The reactions were conducted in 100 mM citrate/phosphate buffer (pH 2.8) containing 0.6 µg MnP-1, 1 mM H_2O_2, and 0.1 mM MCD or 1 mM DMP. Fluoride was supplied as NaF. Values shown here were the mean of triplicates.

Fluoride binding to MnP-1

From the experiments on the fluoride-dependent monochlorodimedone or 2,6-dimethoxyphenol oxidation it is concluded that fluoride plays an important role in these reactions without being a substrate. It is feasible that the effect of fluoride occurs via binding to the enzyme and changing its conformational features to allow oxidation of substrates that are not oxidized by the manganese peroxidases in the absence of fluoride. Fluoride has been reported to be a ligand to the heme co-factor in heme proteins such as MnP of *Phanerochaete chrysosporium* (Sheng and Gold, 1997). To test the fluoride binding to MnP-1, the dissociation constants for fluoride were tested at different pH values (3.4, 4.0 and 4.4) and compared to those for Mn(II).

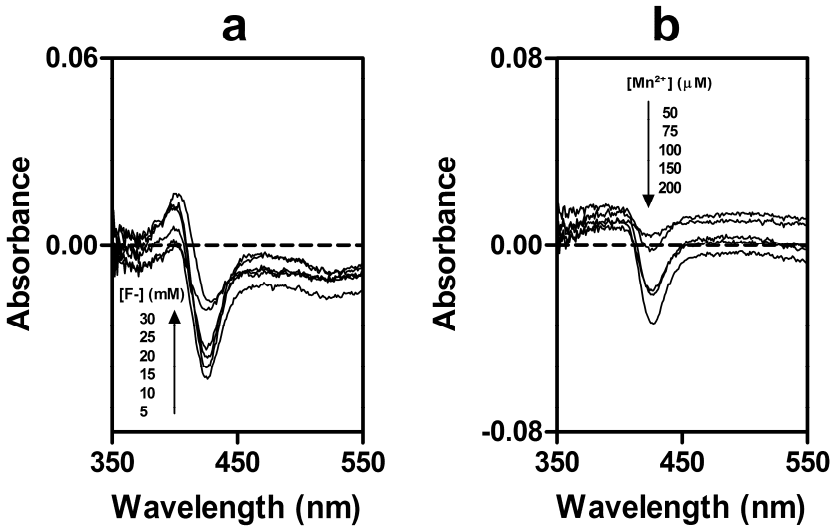

Fig. 3.26 Difference spectra of *B. adusta* strain Ud1 MnP-1 in the presence of NaF (a) or Mn(II) (b). Difference spectra were recorded after addition of Mn(II) or F^- to sample cuvette in 100 mM citrate/phosphate buffer (pH 4.0) at the concentrations indicated.

In cuvettes with citrate/phosphate buffer, the absorption spectra of MnP-1 were recorded in the absence and presence of different Mn(II) or F^- concentrations. From the spectra in the presence of the ions the spectra without addition of ions were subtracted; difference spectra were obtained and depicted for F^- (Fig. 3.26a) and Mn(II) (Fig. 3.26b), respectively. With both ions, the difference spectra showed an absorption maximum at 405 nm and an absorption minimum at 425 nm.

The apparent dissociation constants (K_D) were calculated according to the following equation:

$$1/\Delta A = K_D /\Delta A_\infty \times 1/[S] + 1/\Delta A_\infty \quad \text{(Equation 1)}$$

where ΔA_∞ is the maximal absorption difference obtained upon titration of MnP-1 indicating the

saturation of the enzyme with the ions. [S] is the F^- or Mn(II) concentration in the cuvette. K_D and ΔA_∞ could be calculated from slope and intercept of the double-reciprocal plot of ΔA versus [S] (Fig. 3.27a, b).

From the change in the absorption spectrum, it was deduced that the ions bound to the heme or to the protein close to this cofactor. The stoichiometry of F^- or Mn(II) binding to MnP-1 in the vicinity of the heme was calculated from the logarithmic form of the Hill equation shown below:

$$\log[\Delta A /(\Delta A_\infty - \Delta A)] = h \times \log[S] + \log K_D \quad \text{(Equation 2)}$$

where ΔA_∞ and K_D were calculated from Equation 1 (Fig. 3.27a, b).

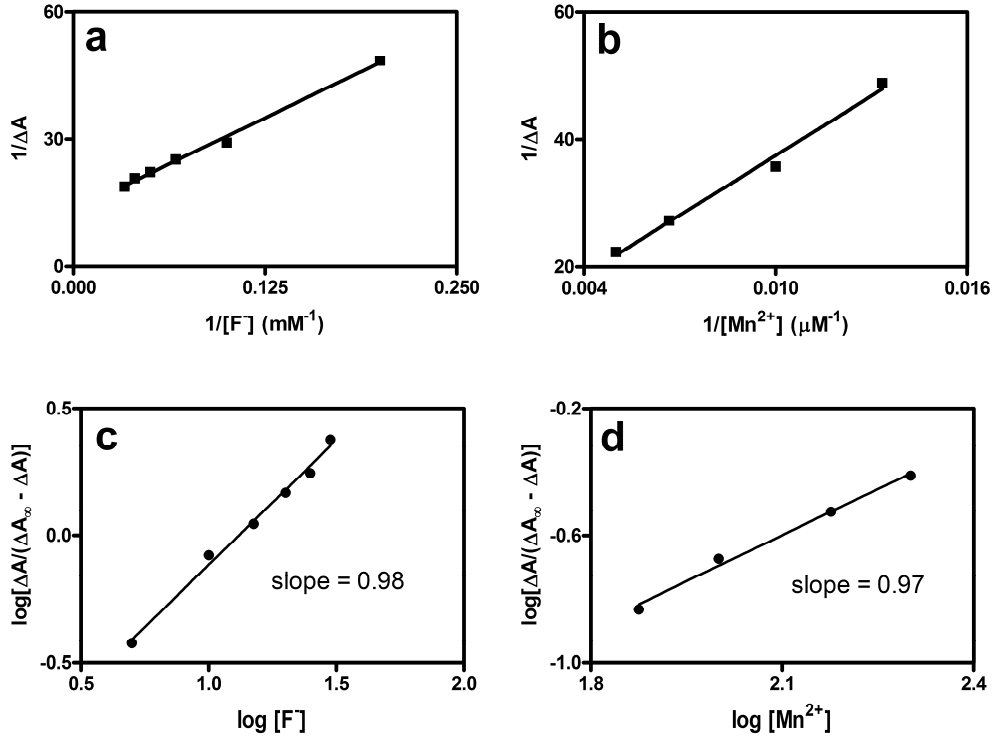

Fig. 3.27 Plot for the calculation of the dissociation constant (a for F^-; b for Mn(II)) and Hill plot for the calculation of the stoichiometry of ion binding to MnP-1 (c for F^-; d for Mn(II)).

A plot of log[ΔA /(ΔA∞ -ΔA)] against log[S] exhibited a straight line with a slope (h) of 0.97 (Fig. 3.27c) and 0.98 (Fig. 3.27d) for Mn(II) and F^-, respectively, indicating the binding of a single atom of F^- or Mn(II) to the heme or its vicinity of the enzyme.

The dissociation constant K_D for F^- was lower at lower pH (Table 3.12). It should be noted that the pK_a of HF dissociation is about 3.2 (Thibodeau, 1985), which is close to the pH at which the K_D value was the lowest. Opposite effect of pH on Mn(II) binding to the enzyme was observed; the K_D for Mn(II) was lower at higher pH values. At pH 3.4, no spectral change was observed at 30 mM

Mn(II) indicating a very high K_D value. The K_D values shown in Table 3.12 also indicate a higher affinity of the enzyme to Mn(II) than to fluoride, which is consistent with the finding that by far higher concentrations of fluoride than of Mn(II) were required for e. g. 2,6-dimethoxyphenol oxidation (see above, Fig. 3.19).

Table 3.12 K_D value of Mn(II) and F^- at different pH

Ligand	K_D value at different pH (mM)		
	3.4	**4.0**	**4.4**
Mn(II)	-	0.5	0.03
F^-	2.5	13.0	565.00

Competition between Mn(II), fluoride and hydrogen peroxide in MnP-1 reactions

Manganese peroxidases were believed to oxidize phenolic substrates only in the presence of Mn(II) (Urzua *et al.*, 1995). Here it is shown that to some extent, fluoride may replace Mn(II) in this respect. It is feasible that fluoride and Mn(II) might compete for the same binding site in the enzyme. To investigate a potential competition of the two ions, monochlorodimedone (MCD) was used as a substrate, since its conversion is strictly fluoride-dependent and manganese-independent; effect of fluoride on Mn(II) oxidation was also investigated.

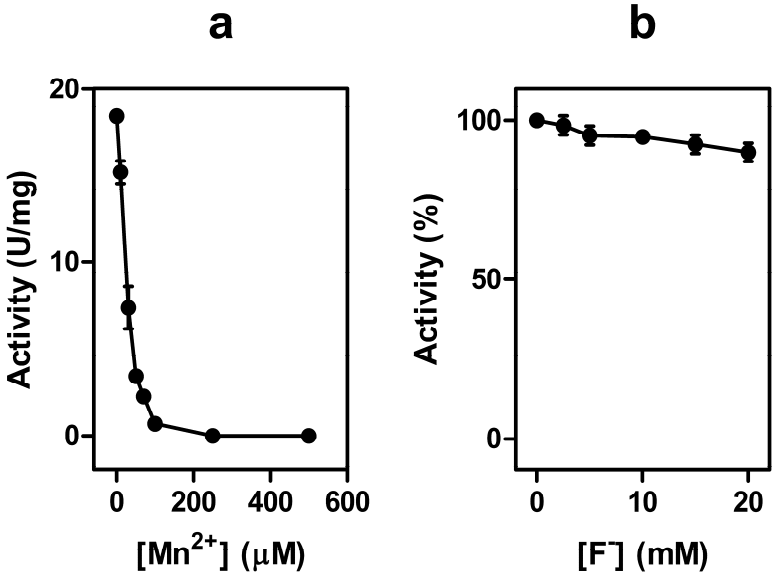

Fig. 3.28 Effect of Mn(II) on fluoride-dependent MCD consumption (a) and effect of fluoride on Mn(II) oxidation (b). a. Reaction mixtures contained 0.6 µg/ml MnP-1 of *B. adusta* strain Ud1, 20 mM F^- and 1 mM H_2O_2 in 100 mM citrate/phosphate buffer, pH 2.8. b. Reaction mixtures contained 0.6 µg/ml MnP-1 of *B. adusta* strain Ud1, 0.5 mM Mn(II) and 0.2 mM H_2O_2 in 50 mM malonate, pH 4.5.

Mn(II) was found to severely inhibit the fluoride-dependent MCD conversion (Fig. 3.28a). At first glance, this result supports the idea of a competition of F^- and Mn(II) for the same binding site in the enzyme. However, fluoride did not significantly inhibit Mn(III)-malonate formation, even when applied in concentrations as high as 20 mM in the presence of 0.5 mM Mn(II) (Fig. 3.28b). The latter result argues against a competition of the ions for the site at which Mn(II) binds as a substrate. The inhibitory effect of Mn(II) on the fluoride-dependent MCD conversion might thus be explained by a competition of Mn(II) and MCD as electron donors for H_2O_2 reduction.

These results indicate a conformational change of the enzyme caused by the binding of fluoride. This might have an effect on the binding of hydrogen peroxide or Mn(II) to the enzyme. This was studied by the determination of the apparent K_m values for H_2O_2 and Mn(II) in the MnP assay in the absence and presence of different concentrations of fluoride (Fig. 3.29).

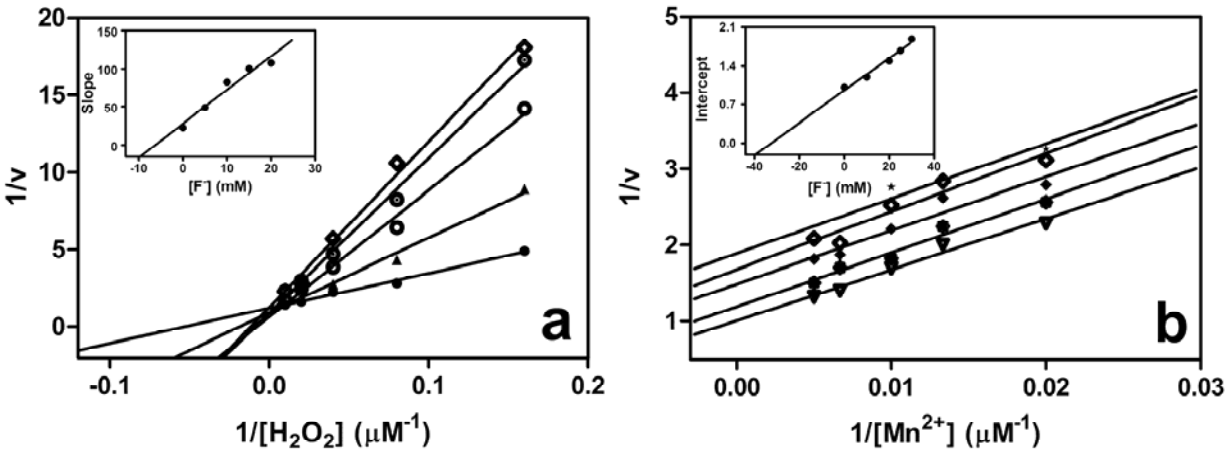

Fig. 3.29 Influence of fluoride on the kinetic constants of MnP-1 for hydrogen peroxide (a) and Mn(II) (b). Lineweaver-Burke plots are shown. The rate of Mn(III)-malonate formation was monitored at 270 nm. a. The reaction mixtures contained 0.6 µg/ml MnP-1 of *B. adusta* strain Ud1, 0.5 mM Mn(II), 50 mM malonate buffer pH 4.5. NaF concentrations of 0 (●), 5 (▲), 10 (○), 15 (⊙), and 20 (◇) mM were used. Inset: replot of the slopes of the Lineweaver-Burke plots vs. [F^-]. b. The reaction mixtures contained 1µg/ml MnP-1 of *B. adusta* strain Ud1, 0.1 mM H_2O_2, 50 mM malonate buffer pH 4.5, Mn(II) was applied in form of $MnSO_4$. NaF concentrations of 0 (▽), 10 (⋇), 20 (◆), 25 (◇), and 30 (⋆) mM were used. Inset: Replot of 1/Vmax vs. [F^-].

For the dependence of the reaction rates of Mn(II) oxidation on the H_2O_2 concentration, Lineweaver-Burk plots at varied concentrations of NaF were linear with a common ordinate-intercept (Fig. 3.29a), indicating that fluoride was a competitive inhibitor for hydrogen peroxide in MnP-1 catalyzed Mn(III)-malonate formation, as has already been indicated by the apparent K_m and K_i values of hydrogen peroxide in the fluoride-dependent MCD consumption (Table 3.5, 3.10).

A K_i of 6.3 mM was calculated from the replot of the slope (App. K_m / V_{max}) vs. [F^-] (Fig. 3.29a, inset).

The Lineweaver-Burk plots of Mn(III)-malonate formation with varying concentration of Mn(II) are shown in Fig. 3.29b. These plots at different fluoride concentrations were linear and parallel, indicating that fluoride is an uncompetitive inhibitor of Mn(II). A K_i of 32.5 mM was calculated from the replot of the intercepts (1/ V_{max}) vs. [F^-] (Fig. 3.29b, inset).

3.3 Model system for the interaction between aerobic fungi and anaerobic bacteria in halogenation/dehalogenation processes

3.3.1 Anaerobic degradation of an organohalogen presumably produced by fungi

Since organohalogens are produced by fungi and found in the environments (de Jong and Field, 1997), it is assumed that halogenating fungi and dehalogenating anaerobes may play an important role in the halogen cycle. To check if in principle anaerobic bacteria can utilize fungal metabolites as substrates, 2,6-dichloroanisole, a natural organohalide presumably produced by fungi (Hjelm *et al.*, 1996) was fed to the organohalide respirer *Desulfitobacterium hafniense* strain DCB-2.

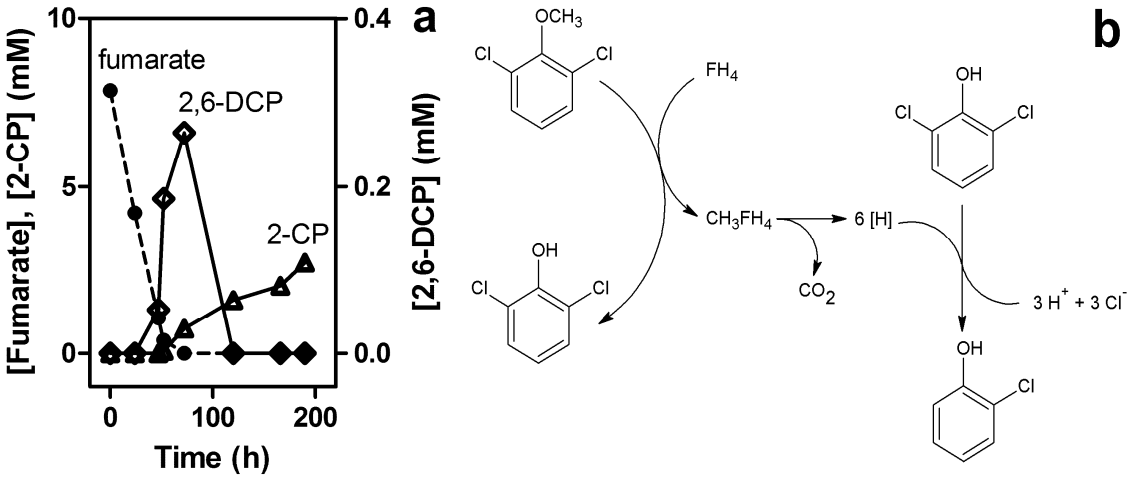

Fig. 3.30 Degradation of 2,6-dichloroanisole by *D. hafniense* strain DCB-2 (a) and tentative scheme of coupling of *O*-demethylation and dehalogenation during this process (b). 2,6-DCP, 2,6-dichlorophenol; 2-CP, 2-chlorophenol. 8 mM fumarate was added initially to start the 2,6-dichloroanisole consumption.

2,6-dichloroanisole (2,6-DCA) was first *O*-demethylated to 2,6-dichlorophenol (2,6-DCP), which was subsequently dehalogenated to 2-chlorophenol (2-CP) (Fig. 3.30a). Concentrations of

2,6-DCA are not shown, since this compound was added in a hexadecane phase due to its low solubility in the medium. No degradation was observed in the absence of fumarate, whereas with 8 mM fumarate to initiate *O*-demethylation of this compound, formation of the dehalogenated product 2-CP was observed even after depletion of fumarate. Seemingly the *O*-demethylation of 2,6-DCA was coupled to the dehalogenation of 2,6-DCP during this time period, with the electrons released from *O*-demethylation flowing to dehalogenation (Fig. 3.30b).

As a control, 2,6-dichlorophenol was used as the substrate instead of 2,6-dichloroanisole. Neither consumption of 2,6-DCP nor production of 2-chlorophenol was observed, which is consistent with the assumption that the dehalogenation of 2,6-DCP utilized the electrons released during the *O*-demethylation from 2,6-DCA to 2,6-DCP.

This experiment shows that in principle *O*-demethylation could be coupled to dehalogenation, using phenyl methyl ethers as electron donor and halogenated phenols as electron acceptor.

3.3.2 Production of halogenated compounds by *Bjerkandera adusta* strain Ud1 and their degradation by *Desulfitobacterium hafniense* strain DCB-2

Bjerkandera adusta has been reported to produce organohalogens (Verhagen *et al.*, 1996). High nitrogen content medium cultures (glucose and peptone as carbon and energy sources) and wood cultures of *B. adusta* strain Ud1were analyzed for the production of halogenated metabolites.

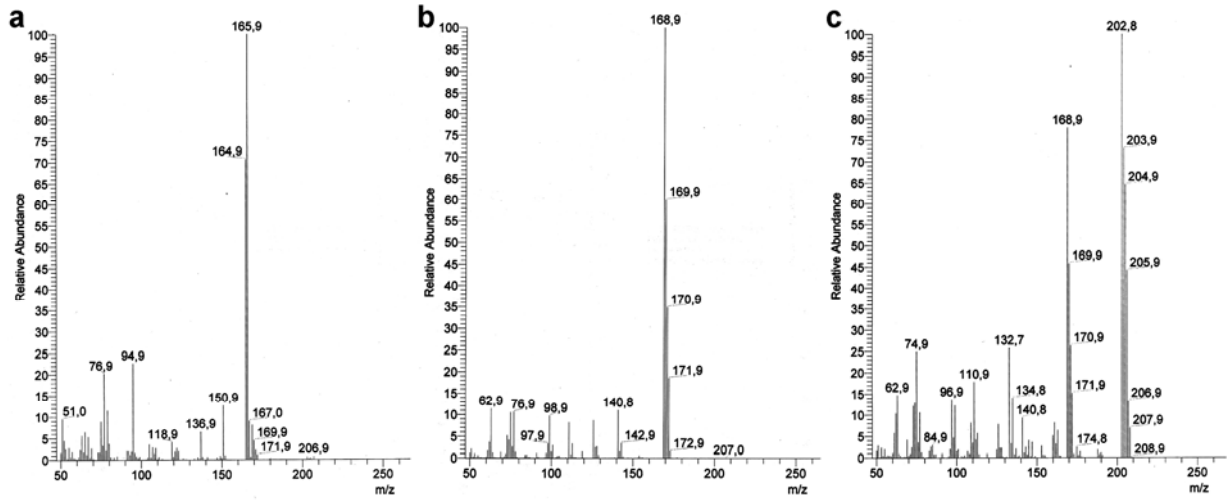

Fig. 3.31 Mass spectra of the *B. adusta* strain Ud1 products. a, veratraldehyde; b, 3-Cl-*p*-anisaldehyde; c, 3,5-dichloro-4-methoxybenzaldehyde.

HPLC analysis using internal standards and GC-MS revealed veratraldehyde and 3-Cl-*p*-anisaldehyde as the predominant products in the ethyl acetate extracts of both cultures (shown for mass spectra in Fig. 3.31a, b). In addition, GC-MS also showed the production of a minor amount of 3,5-dichloro-4-methoxybenzaldehyde (Fig. 3.31c). The mass spectrum was essentially the same as that described earlier for this compounds (de Jong *et al.*, 1994a).

Since 3-Cl-*p*-anisaldehyde was produced by *B. adusta* strain Ud1 as a major halogenated metabolite, it was tested as a substrate for *D. hafniense* DCB-2. With pyruvate as energy and carbon source, 3-Cl-*p*-anisaldehyde was *O*-demethylated and oxidized to 3-Cl-4-hydroxybenzoate or reduced to 3-Cl-4-hydroxybenzyl alcohol (Fig. 3.32), as identified by GC-MS. No dehalogenation was observed.

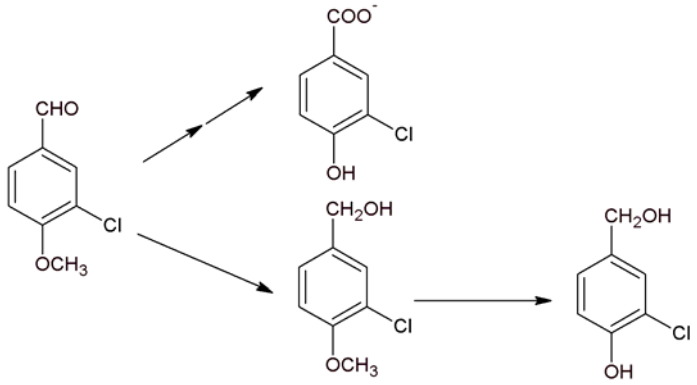

Fig. 3.32 Conversion of 3-Cl-*p*-anisaldehyde by *D. hafniense* strain DCB-2. 40 mM pyruvate was used as energy and carbon source.

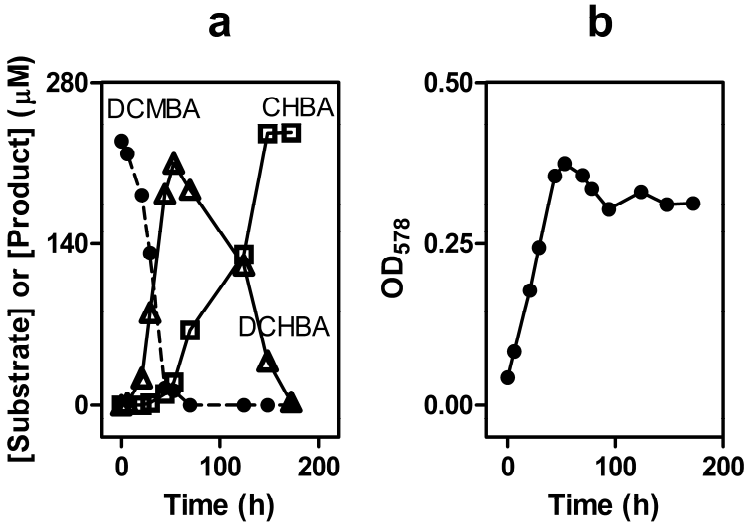

Fig. 3.33 Conversion of 3,5-dichloro-4-methoxybenzoate by *D. hafniense* strain DCB-2. a. Conversion of the substrate; b. growth of *D. hafniense* DCB-2. 40 mM pyruvate was used as energy and carbon source. DCMBA, 3,5-dichloro-4-methoxybenzoate; DCHBA, 3,5-dichloro-4-hydroxybenzoate; CHBA, 3-Cl-4-hydroxybenzoate.

3,5-dichloro-4-methoxybenzoate, the oxidized form of the other fungal halogenated metabolite 3,5-dichloro-4-methoxybenzaldehyde, was also tested for its conversion by *D. hafniense* strain DCB-2. It was first *O*-demethylated to 3,5-dichloro-4-hydroxybenzoate and subsequently dechlorinated to 3-Cl-4-hydroxybenzoate in the presence of pyruvate (Fig. 3.33).

3.3.3 Enrichment of anaerobic bacteria with *O*-demethylating and dehalogenating activities from forest soil

In the experiments described above (Section 3.3.2), a simple halogen cycle could be simulated under laboratory conditions using a halogenating fungus and a dehalogenating anaerobe. To test for the co-existence of these microorganisms in nature, a soil sample was taken from the forest (8-10 cm beneath a moldy spruce litter layer). It was black in color and had a neutral pH. Proteins were extracted from soil stored at -20°C after thawing and measured for ligninolytic enzyme activities. Manganese peroxidase, laccase and Mn-independent peroxidase activities were found (Table 3.13), indicating the presence of lignin degrading fungi. Freezing and thawing might have led to a decrease of the activity so that the actual enzyme activities in native soil might be higher.

Table 3.13 Ligninolytic enzyme activities measured from protein extract of forest soil

Ligninolytic enzyme	Activity (U/kg soil)
Laccase	5
Mn-independent peroxidase	85
Mn peroxidase	50
lignin peroxidase	0

The soil sample was also subjected to anaerobic enrichment cultivation, using 2,6-dichloroanisole followed by the combination of vanillate plus 3-chloro-4-hydroxy-phenylacetate (ClOHPA) as substrates. As shown in Fig. 3.34, a mixed culture with both dechlorination and *O*-demethylation activities was obtained after several passages (4 passages on 2,6-dichloroanisole followed by 2 passages on vanillate plus ClOHPA with 10% inoculum). Furthermore, the bacteria could grow (Fig. 3.34a) upon dehalogenation of ClOHPA (Fig. 3.34b) and/or *O*-demethylation of vanillate (Fig. 3.34c). The mixed culture at this stage was dominated by endospore-forming rod-shaped bacteria; however, it is still not clear if there was one or more bacterial species that were responsible for the

dehalogenation and *O*-demethylation processes.

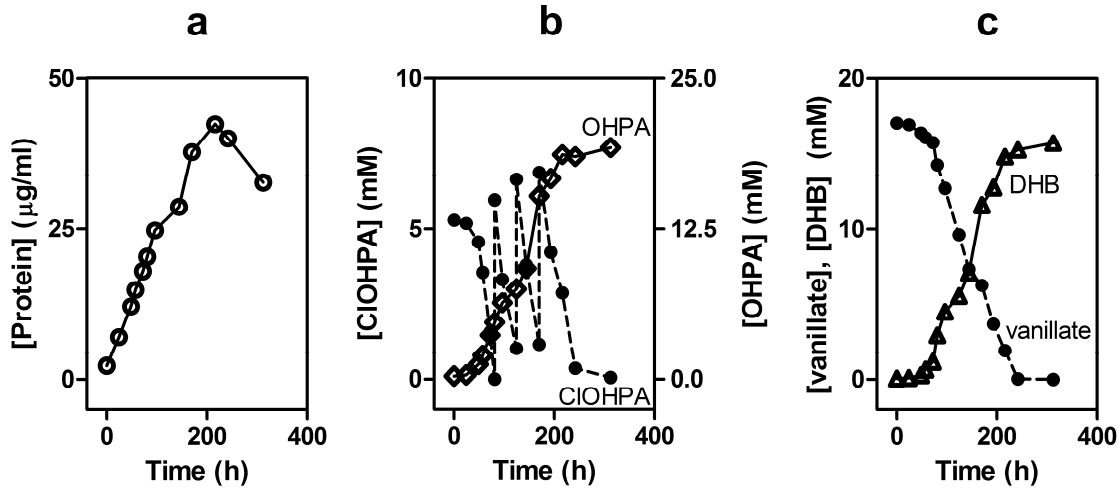

Fig. 3.34 *O*-demethylation and dehalogenation by an anaerobic enrichment culture from forest soil. a. Growth; b. dehalogenation of 3-Cl-4-hydroxy-phenylacetate; c. *O*-demethylation of vanillate. ClOHPA, 3-Cl-4-hydroxy-phenylacetate; OHPA, 4-hydroxy-phenylacetate; DHB, 3,4-dihydroxy- benzoate.

A bacterial isolate capable of both *O*-demethylation and dehalogenation was obtained from this enrichment culture. The 16S ribosomal DNA was amplified from this bacterium and sequenced (not shown). Using this 16S rDNA fragment as query sequence, a maximal identity of 99% with 100% coverage was found with *D. hafniense* strain DCB-2 (Accession number: CP001336.1) via blasting in the National Center for Biotechnology Information (NCBI) database. Growth of this bacterium was observed upon *O*-demethylation of vanillate and dehalogenation of 3-Cl-4-hydroxy-phenylacetate (data not shown).

Lignin degrading fungi and *O*-demethylating/dehalogenating anaerobic bacteria obviously coexisted in the same forest soil sample derived from an unpolluted area. This observation points to a physiological connection between microbial halogenation and dehalogenation in nature.

4. DISCUSSION

4.1 Reductive debromination by reductive PCE dehalogenases

Reductive debromination of brominated ethenes was studied with whole cells and purified reductive dehalogenases from *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* strain PCE-S. Despite of the abundance and variety of brominated compounds found in nature or from anthropogenic sources, biological debromination has been long ignored. Bromoethene dehalogenation by the reductive tetrachloroethene dehalogenase (PCE dehalogenase) was used as a model to study debromination and to gain more information about the substrate spectrum of the dehalogenase in comparison to dechlorination.

The halogenated ethenes studied here have been reported to be soil and groundwater contaminants (Patterson *et al.*, 2007; Grigoriadou *et al.*, 2008) and are shown to be toxic to the organisms and to be possible carcinogens, mutagens and central nervous system depressants (Benya *et al.* 1982; Canton and Wegman, 1983; Cohen *et al.*, 2009). Besides their anthropogenic sources, 1,2-dibromoethenes and tribromoethene have recently been reported as natural organohalogens produced by marine algae (Marshall *et al.*, 1999).

In this study as well as other studies (Wu and Wiegel, 1997; Bedard and Van Dort, 1998; Boyle *et al.*, 1999; Voordeckers *et al.*, 2002) available on the microbial dehalogenation of brominated compounds, bromine substituents seem to be easier removable than chlorine substituents. This may be partially due to the more labile nature of the carbon-bromine bond (Vogel *et al.*, 1987; Allard and Neilson, 2003); also, debromination is thermodynamically more favorable than dechlorination (Dolfing, 2003). This is in accordance with the finding that the purified PCE dehalogenase of *S. multivorans* is able to mediate the dehalogenation of e. g. dibromoacetate faster by a factor of >50 than of dichloroacetate (Neumann *et al.*, 2002). The dehalogenation of dibromoacetate even occurs at much higher rates when the enzyme is heat-inactivated, pointing to an abiotic dehalogenation reaction mediated by the corrinoid cofactor of the PCE dehalogenase. For the experiments reported earlier with sediments, enrichment cultures and mixed cultures, an abiotic or cometabolic rather than metabolic debromination of the brominated compounds therefore cannot be excluded. To our knowledge, the study presented here is the first to describe enzymatic rather than abiotic

debromination with a purified enzyme system.

4.1.1 1,2-dibromoethene (DBE) as an organohalide respiration substrate coupled to bacterial growth

Here for the first time, brominated ethenes were found to be an organohalide respiratory substrate for pure cultures of dehalogenating anaerobes. *S. multivorans* and *D. hafniense* PCE-S were chosen as typical representatives of gram negative and gram positive dehalogenating anaerobes, respectively. As reported previously, *S. multivorans* is able to use a variety of substrates e. g., pyruvate, formate and hydrogen as electron donor for dehalogenation (Scholz-Muramatsu *et al.*, 1995), whereas *D. hafniense* PCE-S can dehalogenate with pyruvate but not formate or hydrogen as electron donor. The growth stimulation by 1,2-dibromoethene (DBE) upon fermentation of pyruvate may be explained by organohalide respiration, i. e. ATP synthesis coupled to reductive debromination of DBE. It is, however, also feasible that DBE just serves as an electron sink for pyruvate oxidation, thus enabling substrate level phosphorylation upon pyruvate oxidation to acetate and CO_2. *S. multivorans* rather than *D. hafniense* PCE-S can utilize formate as an electron donor for respiratory growth with chlorinated ethenes. Since the oxidation of formate cannot be coupled to substrate level phosphorylation, growth on formate plus a halogenated electron acceptor would prove organohalide respiration. Therefore, the growth of *S. multivorans* with formate as electron donor and DBE as electron acceptor presents evidence that the reductive debromination of DBE is coupled to ATP synthesis via organohalide respiration in this organism.

Dechlorination coupled to energy reservation has been reported for two decades. *Desulfomonile tiedjei* was the first anaerobe found to be able to couple the reductive dehalogenation reaction to microbial growth (DeWeerd *et al.*, 1990). Since then, a lot of organohalide respiring anaerobic bacteria were isolated and characterized, including isolates belonging to the genera *Anaeromyxobacter*, *Desulfitobacterium*, *Sulfurospirillum*, *Desulfomonile*, *Desulfuromonas*, *Desulfovibrio*, *Dehalococcoides*, *Dehalobacter* and *Trichlorobacter*. Their substrate spectra spread over a large variety of mono- and polyhalogenated organohalides, such as chlorinated alkanes and alkenes, halogenated benzenes, and phenols, to polychlorinated biphenyls and dioxins (for a recent review, see Smidt and de Vos, 2004).

However, reports on anaerobic organobromide respiration are rare (Boyle *et al.*, 1999). In one recent report (Ahn *et al*., 2003), the conclusion was drawn from the putative presence of dehalogenase genes and is therefore not convincing. The study presented here is the first to describe dehalogention of brominated ethenes by anaerobes which is clearly coupled to energy metabolism.

However, the growth yield per bromine removed calculated from DBE debromination was surprisingly high. As reported earlier for PCE dechlorination (Scholz-Muramatsu *et al.*, 1995), 1.4 g cells (dry weight) was produced by the removal of one mol chlorine, which is also supported by our results. When PCE is dechlorinated by *S. multivorans*, two chlorines are removed from one molecule, whereas the debromination of DBE by the same organism only releases one bromine per molecule. About 20 g cells per mol bromine removed was calculated, indicating that the ATP yield of debromination is much higher than that of dechlorination. This growth yield was reproducible, although there is so far no explanation for that.

4.1.2 Comparison of dehalogenation of halogenated ethenes and propenes

Although neither *S. multivorans* nor *D. hafniense* PCE-S can dechlorinate dichloroethene as depicted in Fig. 4.1, both organisms were found to be able to debrominate dibromoethenes, the brominated analogues of dichloroethenes, either to vinyl bromide or ethene.

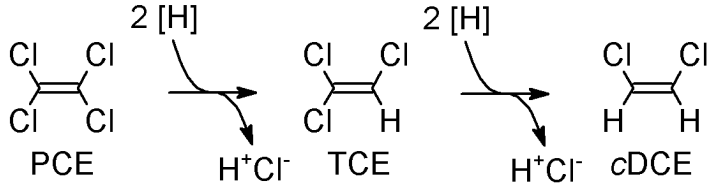

Fig. 4.1: Tetrachloroethene (PCE) reductive dehalogenation via trichloroethene (TCE) to *cis*-1,2-dichloroethene (*c*DCE) mediated by the PCE dehalogenases of *S. multivorans* and *D. hafniense* PCE-S.

Previous studies on the substrate spectrum of the PCE dehalogenase of *S. multivorans* revealed an abiotic dehalogenation activity of the corrinoid cofactor besides the dehalogenation reactions mediated exclusively by the native enzyme (Neumann *et al.*, 2002). Halogenated compounds such as trichloroacetate and dibromoacetate are abiotically dehalogenated by the corrinoid cofactor of the enzyme (see also Glod *et al.*, 1997; Woods *et al.*, 1999; Ruppe *et al.*, 2004) and are

therefore also converted by the heat-inactivated enzyme. However, the dehalogenation of halogenated alkenes such as tetrachloroethene (PCE), trichloroethene, or chlorinated propenes as well as their brominated analogues e. g. tribromoethene (TBE), dibromoethene (DBE) strictly requires the native enzyme.

For chlorinated propenes, a radical mechanism for the reductive dechlorination has been discussed (Schmitz *et al.*, 2007). It was suggested that the mechanism for reductive dehalogenation reactions mediated by the PCE dehalogenase may be different for chlorinated ethenes and chlorinated propenes. This hypothesis is based mainly on two findings: 1. The reactions exhibit different kinetic properties depending on the substrates; e. g. the reductive PCE dechlorination is significantly stimulated by ammonium ions, whereas no effect of ammonium salts on the dehalogenation of chlorinated propenes was observed. 2. Radical reaction intermediates of chloropropene conversion form adducts with the reduced methyl viologen (MV) radical, thus leading to a loss of MV, which can no longer be re-reduced with e. g. Ti(III) citrate. When chlorinated ethenes are used as substrates, MV can be completely re-reduced (Schmitz *et al.*, 2007). Hence, the reaction mechanism for the enzymatic reductive dehalogenation of halogenated ethenes might occur via a mechanism not involving free radical intermediates.

Our experiments with brominated propenes and ethenes revealed that halogenated ethenes did not appear to form stable radical reaction intermediates as indicated by the complete re-reduction of MV, while with halogenated propenes part of the MV was lost probably due to adduct formation. This difference between the conversion of halogenated ethenes and propenes was independent on the halogen type. In addition, ammonium ions significantly stimulated the dehalogenation of chlorinated or brominated ethenes, whereas no stimulating effect of ammonia was observed for the dehalogenation of halogenated propenes.

These experiments led to the conclusion that the type of halogen substituent is less important for the reaction mechanism than the carbon backbone. It is feasible that the allylic radical intermediate which might be formed in the course of the dehalogenation of halogenated propenes is rather stable; hence, the reductive debromination and dechlorination of halogenated propenes follow the same reaction mechanism. It should be noted, however, that the indications for one or the other reaction mechanism are indirect and the conclusions are drawn in analogy to earlier experiments (Schmitz *et al.*, 2007).

The enzymatic studies on the conversion of 1,2-dibromoethene (1,2-DBE) indicate that in principal the dehalogenation of dihalogenated compounds by the enzymes is possible. However, the exclusive formation of the dichloroethene (DCE) *cis*-isomer as the end product was reported with several PCE reductive dehalogenases isolated from phylogenetically different bacterial species (for a review see Holliger *et al.*, 2003). The reason for the prevention of a further enzymatic *c*DCE dechlorination is not known. *Trans*-1,2-dichloroethene or 1,1-dichloroethene are also reported to be not converted by *S. multivorans* (Neumann *et al.*, 1995). The complete dechlorination of *c*DCE to vinyl chloride and ethene was only described for the partially purified trichloroethene reductive dehalogenase of *Dehalococcoides ethenogenes* 195 (Magnuson *et al.*, 2000).

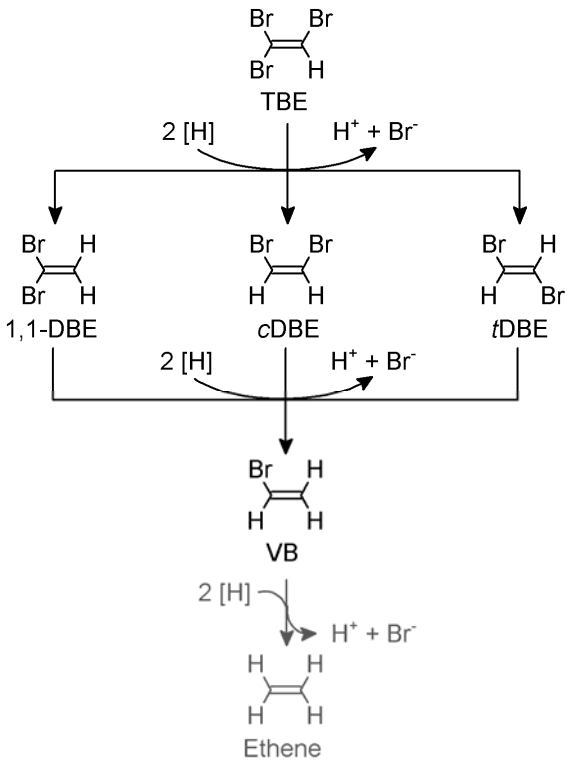

Fig. 4.2 Dehalogenation of tribromoethene by PCE dehalogenase of *S. multivorans* and *D. hafniense* PCE-S. In gray: only mediated by the PCE dehalogenase of *D. hafniense* PCE-S.

The formation of different isomers of DBE from tribromoethene (TBE) (Fig. 4.2) might at first glance point to an external electron transfer radical reaction as proposed for the conversion of halogenated propenes. However, no radical reaction intermediates interacting with the methyl viologen radical appear to be formed *in vitro*, arguing against this mechanism. Hence, the dehalogenation of dibrominated ethenes by the PCE dehalogenase brings up further questions on the

reaction mechanism of reductive dehalogenation mediated by the enzyme. Possibly, the activation of the carbon-halogen bond is the reason for the limited ability to dehalogenate chlorinated ethenes as compared to brominated ethenes.

As shown in Fig. 4.2, although both PCE dehaloeganses of *S. multivorans* and *D. hafniense* PCE-S were able to debrominate tribromoethene and dibromoethenes, there was a significant difference between the debromination products. VB was the final product of TBE or DBE debromination with the PCE dehalogenase of *S. multivorans*, whereas a significant amount of ethene was also measured with the PCE dehalogenase of *D. hafniense* PCE-S, pointing to a more effective debromination ability of the PCE dehalogenase of *D. hafniense* PCE-S, regardless of a lower specific activity, compared to that of *S. multivorans*.

To conclude, organohalide respirers such as *S. multivorans* or *D. hafniense* PCE-S appear to perform three categories of reductive dehalogenation reactions depending on the substrate: 1. dehalogenations coupled to growth and energy conservation; 2. enzymatic dehalogenations not coupled to energy conservations, but mediated exclusively by the native enzyme and may support growth by providing an electron sink; 3. abiotic dehalogenation by cofactors with appropriate electron donors. The substrates PCE, TCE, and 1,2-DBE fall into the first category. These are also the substrates which are able to induce the PCE dehalogenase in PceA-depleted cells. In addition, the reduction of these substrates was stimulated by ammonium ions in *S. multivorans* and a formation of adducts from reduced methyl viologen and potential radical intermediates was not observed. Chlorinated propenes belong to the second category. Their conversion was not stimulated by ammonium ions. A significant portion of methyl viologen was irreversibly converted in the enzymatic assay, pointing to an external electron transfer upon formation of a radical intermediate, and the enzyme was not induced by these substrates. Brominated propenes are converted, even though at low rates, by vitamin B_{12} in the absence of protein. The conversion of brominated propenes, however, was much faster with the native enzyme. This indicates that the brominated propenes belong to the second category; however, a slow abiotic conversion is also mediated by corrinoids. Substrates such as trichloroacetate fall into the third category. These compounds are converted by corrinoids at comparable rates to those observed with the native enzyme (Neumann *et al.*, 2002).

4.2 Halide ion-involved reactions by fungal peroxidases

4.2.1 Brominating activity of versatile peroxidases of *Bjerkandera adsuta* strain Ud1

Basidiomycetes capable of producing halogenated metabolites were screened for halogenating peroxidase production. The versatile peroxidases (VPs) of *Bjerkandera adusta* strain Ud1 were found to exhibit brominating activity. No such halogenating activity was described before for the important lignin-decaying *Bjerkandera* species, which have been reported as one of the most potent producers of organohalogens under laboratory conditions as well as in nature (de Jong *et al.*, 1992).

Despite of the abundance of halogenated metabolites produced by basidiomycetes, the enzymes responsible for this *in vivo* halogenation are so far unknown. *In vitro* studies have shown that some peroxidases purified from the lignin-degrading basidiomycetes, e. g. lignin peroxidase (LiP) (Renganathan *et al.*, 1987; Farhangrazi *et al.*, 1992) and manganese peroxidase (MnP) (Sheng and Gold, 1997) from *Phanerochaete chrysosporium* as well as *Agrocybe aegerita* Peroxidase (AaP) from *Agrocybe aegerita* (Ullrich *et al.*, 2004) which was later put to the novel group of aromatic peroxygenases (Pecyna *et al.*, 2009), have in common halogenating activity as side reactions.

The first haloperoxidase described is the chloroperoxidase (CPO) of the ascomycete *Caldariomyces fumago* (Shaw and Hager, 1961). A synthetic substrate monochlorodimedone, which is structurally similar to the natural substrate of this enzyme (2-chloro-1,3-cyclopentanedione) has been since then widely used to assay haloperoxidases from a variety of prokaryotic and eukaryotic organisms. This chloroperoxidase of ascomycete origin was the best studied halogenating enzyme. A vanadium chloroperoxidase, the *v*CPO from the ascomycete *Curvularia inaequalis*, has recently been discussed for its possible involvement in lignin degradation. During colonization of wood by this ascomycete fungus, chlorinated aromatic compounds production as well as *v*CPO production was detected (Ortiz-Bermudez *et al.*, 2007).

The halogenated compounds are supposed to be good substrates for aryl alcohol oxidases which produce hydrogen peroxide for ligninolytic peroxidases (de Jong *et al.*, 1994b). It is feasible that on one hand the ligninolytic peroxidases (MnP, LiP, VP) with halogenating side activity synthesize organohalogens using lignin decomposition products (resulting from their ligninolytic activi-

ties) together with H_2O_2 and halide ions; on the other hand the organohalogens are used as substrates for aryl alcohol oxidases to produce H_2O_2, and the H_2O_2 produced here is in turn used by the ligninolytic peroxidases to catalyze lignin decomposition and halogenation (Fig. 4.3). Considering the readily available halide ions in terrestrial environments, halogenation might therefore be an important process in lignin degradation.

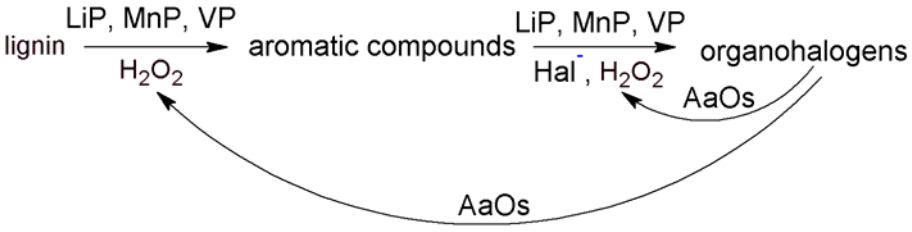

Fig. 4.3 Scheme of putative cycle involving ligninolytic peroxidases with side halogenating activity. MnP: manganese peroxidase; LiP: lignin peroxidae; VP: versatile peroxidase; AaO: aryl alcohol oxidase; Hal^-, halide anions.

Although there is a much higher chloride concentration than that of bromide in terrestrial environments, the ligninolytic peroxidases with halogenating activities described so far are mostly brominating peroxidases, which are unable to oxidize chloride.

Table 4.1 presents a summary of fungal heme peroxidases that are reported to have halogenating activities. The versatile peroxidases studied here are so far the only peroxidases that showed a fluoride-dependent monochlorodimedone (MCD) consumption activity (a seeming "fluoroperoxidase" activity). With all other peroxidases tested, no such activity was observed (Morris and Hager, 1966), although fluoride binding was observed in some cases (Renganathan *et al.*, 1987; Sheng and Gold, 1997).

Compared to the bromination of MCD, in the fluoride-dependent MCD consumption catalyzed by versatile peroxidases of *B. adusta* strain Ud1, the affinity of the enzymes to H_2O_2 was lower and the inhibition of the reactions by other halide ions was less significant. Moreover, in the absence of the substrate (MCD), the heme was rapidly bleached in the presence of bromide and H_2O_2, whereas no enhanced bleaching was observed when bromide was replaced by fluoride. The presence of other halide ion, e. g. chloride, had a less significant effect on the fluoride-dependent MCD consumption than on MCD bromination. The reaction mechanism of fluoride-dependent reactions catalyzed by the versatile peroxidases might therefore be different from that of bromination.

Table 4.1 Occurrence and properties of fungal heme peroxidases with halogenating activity

Organism	Taxonomic Group	Enzyme	Halide	Reference
Bjerkandera adusta	Basidiomycete	Versatile peroxidases (VPs)	Br^-, I^-, (F^-)[1]	This work
Agrocybe aegerita	Basidiomycete	*A. aegerita* peroxidase (AaP)	(Cl^-)[1],Br^-, (I^-)[1]	Ullrich *et al*., 2004; Ullrich and Hofrichter, 2005
Phanerochaete chrysosporium	Basidiomycete	Lignin peroxidase (LiP)	Br^-, I^-	Renganathan *et al.*, 1987 ; Farhangrazi *et al.,* 1992
		Manganese peroxidase (MnP)	Br^-, I^-	Sheng and Gold, 1997
Caldariomyces fumago	Ascomycete	Chloroperoxidase (CPO)	Cl^-, Br^-, I^-	Morris and Hager， 1966

[1]( ), doubtful or low activty.

4.2.2 Fluoride-dependent reactions mediated by MnP-1 of *Bjerkandera adusta* strain Ud1

Besides the versatile peroxidases of *Bjerkandera adusta* strain Ud1 mentioned above, manganese peroxidase (MnP) of *B. adusta* strain Ud1 as well as MnPs of three other fungal species was found to be able to catalyze fluoride-dependent monochlorodimedone (MCD) conversion. In challenge to the formerly assumed Mn dependency of manganese peroxidase, fluoride was for the first time found to induce substrate oxidation activities of MnP in the absence of manganese at acidic pH values. In the presence of fluoride instead of manganese, a series of phenolic substrates as well as MCD were oxidized by MnP-1 of *B. adusta* strain Ud1. The low pH optimum of MnP-mediated oxidation of phenolic compounds or monochlorodimedone in the presence of fluoride might be due to the increase of compounds I and II potentials as the pH decreases, as suggested by Heinfling *et al.* (1998) in case of veratryl alcohol oxidation by versatile peroxdiase.

No hypofluorous acid was formed during the MnP-1 mediated fluoride-dependent reaction, as indicated by the findings that the presence of fluoride did not significantly enhance enzyme inactivation and that no H_2O_2 was consumed during enzyme inactivation. In this respect the fluoride-dependent reaction is significantly different from the haloperoxidase reactions. In the haloperoxidase reaction, reduction of hydrogen peroxide by the enzyme generates a redox potential sufficiently positive to oxidize the halide ion, yielding an electrophilic halogenating reagent, which is generally believed to be hypohalous acid (for a review, see van Pée, 2001). Moreover, peroxi-

dase-catalyzed fluorination could be excluded by analysis of the reaction products. Fluoride was even not a substrate for the enzyme.

The presence of fluoride was however obligatory and the velocity of the fluoride-dependent reactions increased with increasing fluoride concentration. Fluoride was reported to bind with a high affinity to heme enzymes at acidic pH values (Zgliczynski *et al.*, 1983). Fluoride can coordinate to the heme of heme containing peroxidases e. g. horseradish peroxidase, cytochrome c peroxidase as a sixth ligand to form high-spin ferric hexacoordinate species if the sixth coordination position of the heme iron is vacant or occupied by a weak field ligand such as water, causing considerable porphyrin expansion (Mino *et al.*, 1988). Fluoride binding was also reported for the MnP of *Phanerochaete chrysosporium* (Sheng and Gold, 1997). However, no Mn(II)-independent enzyme activity in the presence of fluoride was described for this enzyme. Fluoride has rather been reported as inhibitor for heme-containing proteins (Hager *et al.*, 1966; Murphy and Coll, 1992; Stachowska *et al.*, 2000), either via formation of inactive enzyme-F^- complex (Murphy and Coll, 1993) or via competitive and/or noncompetitive binding to the active site (Stachowska *et al.*, 2000).

^{1}H-NMR experiments suggest that iodide binds to horseradish peroxidase (HRP) at the distal site near the heme iron (Sakurada *et al.*, 1987). The binding site for halide ions in myeloperoxidase has been suggested to be the heme iron or its close vicinity (Zgliczynski *et al.*, 1983). Mutagenesis studies have already shown in heme-containing peroxidases that the distal arginine is important for controlling the ligand binding via a strong hydrogen bond between the positively charged guanidinium group and the anion. The anion and the distal histidine might contribute to the stability of the enzyme-F^- complex by accepting a proton from HF and hydrogen-bonding, through a water molecule, to the anion (Neri *et al.*, 1997). As the conserved amino acids for controlling fluoride binding found in crystal structures of the heme proteins are in the region of the conserved hydrogen peroxide binding pocket (the distal histidine and arginine) (Sundaramoorthy *et al.*, 1994), a competitive binding of hydrogen peroxide and fluoride is assumed. Since the distal cavity structure is highly conserved in all heme peroxidases, fluoride binding should be very similar among different enzymes. We can thus assume there is also a fluoride binding site near to the distal cavity of manganese peroxidase.

The difference spectroscopy showed fluoride bound to MnP-1 of *B.adusta* strain Ud1 in the heme pocket with a stoichiometric ratio of 1:1. Furthermore, fluoride was found to be a competitive

inhibitor for hydrogen peroxide, as indicated by the apparent K_m and K_i values of H_2O_2 in the presence or absence of fluoride as well as the kinetic parameters study of Mn(II) oxidation with fluoride as a competitor. The competitive inhibition of fluoride for reaction with hydrogen peroxide and its uncompetitive inhibition for Mn(II) oxidation indicate that a fluoride binding site is close to the hydrogen peroxide binding site rather than the Mn binding site. However, all the studies on fluoride binding were targeted at its effect on the heme pocket; further fluoride binding far from the heme pocket can therefore not be excluded.

Previous studies have found that fluoride binding causes structural changes in the heme pocket of cytochrome c peroxidase (Edwards *et al.*, 1984). The formation of adduct between cytochrome c peroxidase and F^- leads to the induction of significant changes in the distal cavity. Upon fluoride binding the distal arginine and histidine residues are significantly perturbed and the water molecules present in the active site are rearranged (Edwards and Poulos, 1990). Similar conformational change upon the formation of manganese peroxidase-F^- adduct might have occurred in this study. The fluoride bound to the heme pocket of MnP-1 competitively inhibited H_2O_2 binding via perturbing the distal cavity. If further fluoride binding far away from the heme pocket occurred, the protein structure in the vicinity of this binding site might also be affected, which might lead to some further changes in the catalysis of the enzyme.

Versatile Mn(II)-independent oxidase activities were added to MnP-1 of *B. adusta* strain Ud1 in the presence of fluoride and Mn(II) significantly inhibited the “new” oxidase activities; whereas the Mn(II) oxidation activity was only slightly inhibited by fluoride. This result is similar to that reported for a MnP mutant. A single mutation of the amino acid from serine to tryptophan (S168W) in MnP corresponding to W171 in LiP added veratryl alcohol peroxidase activity to the enzyme without significantly affecting Mn(II) peroxidase activity. This activity was severely inhibited by Mn(II) (Timofeevski *et al*., 1999). Such an inhibition could be explained by the activation of a long-range electron transfer pathway upon the formation of a MnP-F^- adduct or mutation. In the presence of Mn(II), the direct electron transfer from Mn(II) to the heme was preferred, which in turn severely inhibited the long-range electron transfer. The distance dependency of protein electron transfer reactions has been previously reviewed (Onuchic *et al.*, 1992). Since the binding of fluoride competitively inhibits hydrogen peroxide binding and might distract part of the electrons to be passed over the long-range pathway, the Mn(III) formation was partially inhibited by fluoride. Such

long-range electron transfer pathways have been described for lignin peroxidase and versatile peroxidase (Fig. 4.4; Pérez-Boada *et al.*, 2005) and are very common among biological processes, e. g. photosynthesis and respiration (for a review, see Ferguson-Miller *et al.*, 2007). It has been reviewed earlier that the electron transfer kinetics in protein-protein reactions is regulated by the dynamics of conformational changes in the protein-protein complex (Nocek *et al.*, 1996). It is therefore feasible to assume the activation of a long-range electron transfer pathway upon the formation of the enzyme-F^- adduct, where conformational changes might have occurred.

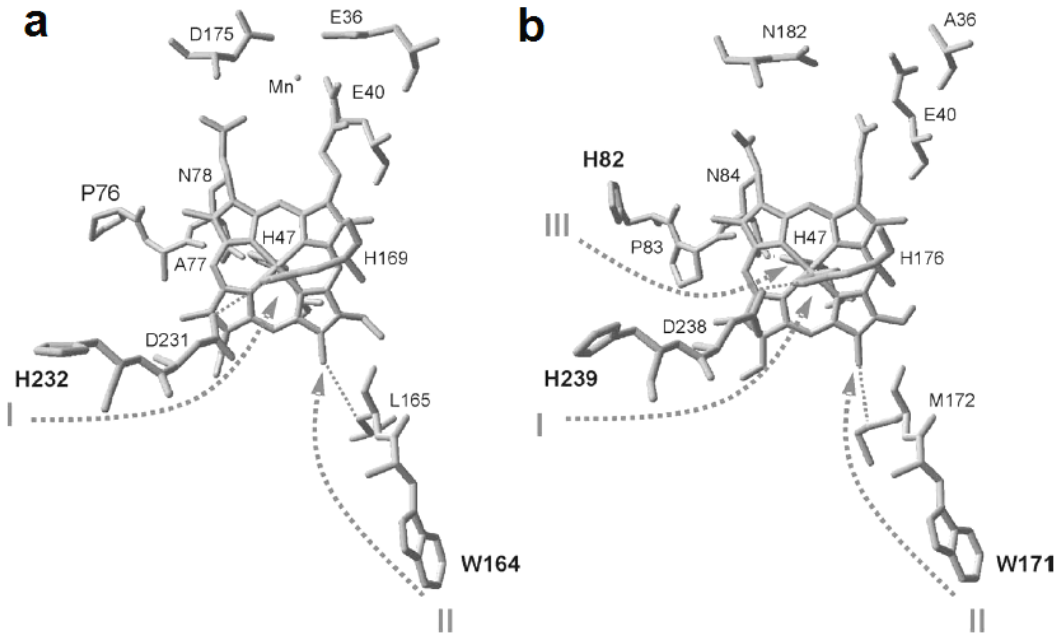

Fig. 4.4 Putative long-range electron transfer (LRET) pathways in versatile peroxidase (VP) and lignin peroxidase (LiP) molecular structures (adapted from Pérez-Boada *et al.*, 2005). Axial view of the peroxidase heme (from proximal His side) showing LRET pathways in *Pleurotus eryngii* VP isoenzyme VPL (from solvent exposed Trp164 and His232) (a) and *Phanerochaete chrysosporium* LiP isoenzyme H8 (from exposed His82, Trp171 and His239) (b).

Despite of a wide variation in the substrate spectrum, most heme peroxidases share the catalytic cycle where steps of one-electron oxidation occur via a three-step reaction process with Compound I and Compound II as intermediates of the enzyme (Sundaramoorthy *et al.*, 1994). The molecular structure is very similar among ligninolytic peroxidases, including a heme cofactor located at an internal cavity, i. e. the heme pocket, which is connected to the protein surface by two small access channels. The larger one is conserved in all heme peroxidases and is used by hydrogen peroxide to reach the heme and react with Fe(III) to form Compound I (the two-electron

activated form of the enzyme). The second channel extends directly to the heme propionates, which are the site where manganese peroxidase and versatile peroxidase oxidize Mn(II) to Mn(III) (Pérez-Boada *et al.*, 2005). It has been shown that the oxidation of some phenols and dyes by versatile peroxidase occurs at the edge of the main heme access channel (for a review, see Ruiz-Duenas *et al.*, 2009a).

Substrate oxidation at the peroxidase surface depends on both substrate affinity and redox potential (Ruiz-Duenas *et al.*, 2001). In the absence of manganese and in the presence of fluoride, e. g. monochlorodimedone and 2,6-dimethoxyphenol were utilized by MnP-1 as direct substrates. The apparent K_m values determined for these compounds in the fluoride-dependent reactions were in the same order of magnitude as for Mn(II), indicating a high affinity of the enzyme to these substrates. As has been described for substrate oxidation by lignin peroxidase, where the reactions are believed to occur at the protein surface via a long-range electron transfer pathway, multiple sites might be responsible for oxidation of different aromatic substrates (Martínez *et al.*, 2002). Site-directed mutagenesis studies have shown that an exposed tryptophan, e. g. Trp164 in versatile peroxidase (Ruiz-Duenas *et al.*, 2009b), Trp171 in lignin peroxidase (Gelpke *et al.*, 2002), is required for the oxidation of veratryl alcohol. Transient-state study of Cd(II) inhibition and site-directed mutagenesis experiments have pointed to a site (or sites) other than Mn binding site for binding phenolic substrates in MnP (Whitwam *et al.*, 1997; Youngs *et al.*, 2000).

The redox potential of the substrate is an inverse function of the length of the electron transfer pathway to heme (Martínez, 2002). In Mn(II) oxidation, direct electron transfer to the heme may be expected, considering the location of the Mn binding site; whereas in the fluoride-dependent reactions, fluoride binding to MnP might have activated the long-range electron transfer pathway and enabled the electrons to be transferred from the oxidation sites of the phenolic compounds or monochlorodimedone on the protein surface to the oxidized heme (Compound I and/or II). The inability of MnP to oxidize nonphenolic aromatic compounds such as 1,2,3,5-tetramethoxybenzene and veratryl alcohol might be due to the relatively low redox potential of this enzyme. The binding of fluoride to the enzyme might have not increased its redox potential since the high redox potential of ligninolytic peroxidases seems to be related to the distance between the heme iron and the proximal histidine (Martínez *et al.*, 2002).

A hypothesis of the reaction mechanism is brought up based on these observations and facts.

Fluoride binding might have altered the structural conformation of the protein close to the binding site, which induced the activation of a long-range electron transfer pathway. This enabled the MnP to oxidize the substrates on the periphery of the enzyme, which have no binding sites near the heme pocket. The fluoride activation of MnP in the absence of Mn sheds some light on the reaction mechanism of MnPs. For the first time, ligand binding is reported to induce a new series of enzyme activities.

Besides fluoride, NO, CO and CN^- were also reported as ligands for heme proteins. The formation of both cytochrome c peroxidase (CCP)-CN^- and CCP-F^- has been shown to influence the conformation of distal amino acid residues and disturb the distal cavity structure of the enzyme (Edwards and Poulos, 1990). It is therefore interesting to test if CN^- has a similar effect on the substrate spectrum of MnP as F^-.

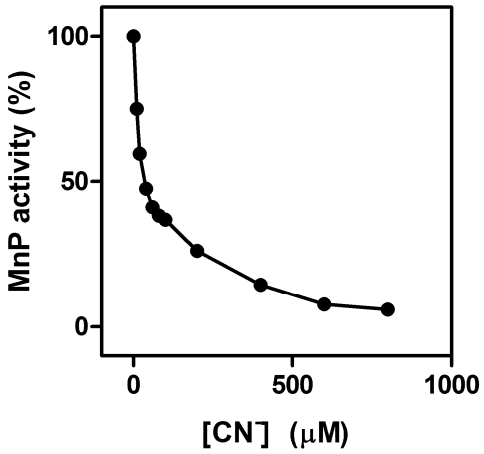

Fig. 4.4 Effect of cyanide on Mn(II) oxidation. Reactions were carried out in 50 mM malonate (pH 4.5) containing 1 µg MnP I of *B. adusta* strain Ud1, 0.5 mM Mn(II), 0.2 mM H_2O_2.

In the presence of cyanide, neither monochlorodimedone consumption nor 2,6-dimethoxyphenol conversion was observed with MnP-1 when Mn(II) was absent (data not shown). Cyanide had a severe inhibitory effect on Mn(II) oxidation (Fig. 4.4). Thus, it seems the catalytic features induced by fluoride binding are specific and can not be replaced by cyanide binding.

As has been reported for cytochrome c peroxidase, in the fluoride-enzyme adduct arginine (Arg)-48 moves in toward the ligand to form a hydrogen bond or ion pair with the fluoride, while in all other complexes formed by the enzyme and ligands such as CN^-, NO and CO, Arg-48 moves away from the ligand (Edwards and Poulos, 1990). This difference might have led to the different behaviors after ligand binding.

Furthermore, the MnP catalyzed monochlorodimedone oxidation in the presence of fluoride leads again to a critical view on the widely applied monochlorodimedone (MCD) assay for haloperoxidases. As suggested by Wagner *et al.* (2008), a negative control without halide ion should be always applied to minimize the false positive reactions caused by unspecific oxidation of MCD by the peroxidase. However, our study shows that this control might not be sufficient to guarantee a truly positive halogenation reaction. A better assay should be developed.

4.3 Interaction between aerobic halogenating fungi and anaerobic dehalogenating bacteria

Although the chlorinating enzymes of *B. adusta* strain Ud1 remain unknown, the *de novo* synthesis of some chlorinated fungal metabolites was observed when growing on lignocellulose substrate (wood chips) or other complex substrates such as glucose-peptone. These halogenated compounds as well as phenyl methyl ethers could serve as substrates for *Desulfitobacterium hafniense* strain DCB-2, a dehalogenating/*O*-demethylating anaerobe (see also Neumann *et al.*, 2004).

D. hafniense strain DCB-2 was able to *O*-demethylate and/or dechlorinate the fungal halogenated metabolites, e. g. 2,6-dichloroanisole, 3-Cl-*p*-anisaldehyde and 3,5-dichloro-4-methoxybenzoic acid. Phenyl methyl ethers are an important group of lignin degradation products, which could be easily derived from the cleavage of C-C or ether bonds in lignin polymer molecule (Boerjan *et al.*, 2003). When halogenated by the fungi, they might become suitable substrates for *O*-demethylation and/or dehalogenation. This indicates that at least in theory an interaction between aerobic halogenating/lignin-degrading fungi and anaerobic dehalogenating/ *O*-demethylating bacteria might occur. The production of organohalogens by lignin degrading fungi has been described (de Jong and Field, 1997), and the study on anaerobic reductive dehalogenation has also attracted extensive attention in the last two decades (for reviews, see Holliger *et al.*, 2003; Smidt and de Vos, 2004). However, links are missing between metabolic activities of the halogenating fungi and dehalogenating bacteria.

Examinations of how anaerobic bacteria contribute to a natural halogen cycle have so far been sparse and primarily restricted to the *in vitro* degradation of chlorinated hydroquinone fungal metabolites (Milliken *et al.*, 2004) and halogenated phenols (Boyle *er al.*, 1999). No anaerobic

dechlorination has been reported so far for the chlorinated anisyl metabolites (CAM) (Verhagen *et al.*, 1998b), despite the fact that it is suggested to be the most ubiquitous and ecologically significant natural organohalogens produced by higher fungi (Field *et al.*, 2003). Concentrations of up to 75 mg/kg in wood and leaf litter have been reported (Gribble, 1995). CAMs are believed to play an important part in fungal lignin degradation, acting as recyclable substrates for key enzymes in the process (de Jong *et al.*, 1994b). 3,5-dichloro-*p*-anisyl alcohol and its oxidized forms are believed to be the most ubiquitously produced CAM and are major metabolites of fungi belonging to the genera *Hypholoma, Pholiota, Stropharia, Lepista, Oudemansiella, Phellinus, Phylloporia, and Bjerkandera* (de Jong and Field, 1997). Especially in *H. fasciculare* cultures, together with its acid form, 3,5-dichloro-*p*-anisyl alcohol accounts for over 99% of the total adsorbable organic halogen (AOX) (Verhagen *et al.*, 1998a). Despite of its abundant production, so far there is only one report on its anaerobic biotransformation, involving demethylation and dimerization. However, the chlorine was not removed by the microbial community tested (Verhagen *et al.*, 1998b).

3,5-dichloro-4-methoxybenzaldehyde was also detected in *B. adusta* strain Ud1 cultures in this study. Its oxidized form, 3,5-dichloro-4-methoxybenzoate, was *O*-demethylated and dechlorinated to 3-Cl-4-hydroxybenzoate by the anaerobic bacterium *Desulfitobacterium hafniense* strain DCB-2. *O*-demethylation occurred prior to dechlorination, as observed here for the degradation of 2,6-dichloroanisole by the same bacterium. As described for the anaerobic conversion of 2,6-dichloroanisole, *O*-demethylation could have served as an electron donating process for the reductive dehalogenation. The other halogenated metabolite detected in *B. adusta* strain Ud1 cultures was 3-Cl-*p*-anisaldehyde, which is another frequently occurring chlorinated aryl metabolite of fungal source (de Jong and Field, 1997). LC-MS analysis showed it was converted to 3-Cl-4-hydroxybenzoate or 3-Cl-4-hydroxybenzyl alcohol via oxidation or reduction and *O*-demethylation.

For all substrates tested, the chlorine substituent at the ortho-position to the hydroxyl group was not removable by *D. hafniense* DCB-2 when the aromatic ring was monochlorinated (2-chlorophenol, 3-Cl-4-hydroxybenzoate or 3-Cl-4-hydroxybenzyl alcohol). However, the same bacterium was able to dechlorinate 3-Cl-4-hydroxy-phenylacetate (Christiansen *et al.*, 1996), so the ortho-hydroxyl group does not seem to be the barrier for further dechlorination. Probably a methyl group at the para-position facilitates the removal of the chlorine substituent.

Since the anaerobic bacteria were found to be able to *O*-demethylate and dehalogenate the halogenated phenyl methyl ethers produced by aerobic lignin degrading fungi, there might be a potential interaction between these microorganisms in natural environments, e. g. flooded soil, rotten wood or any other environmental change which turns the oxic niches to anoxic conditions (Milliken *et al.*, 2004). However, no evidence for a physical interaction between these two microbial groups has been reported before.

The distribution of *Desulfitobacterium* spp. in various types of soils has been reported (Lanthier *et al.*, 2001). It is suggested that the ubiquitous distribution of *Desulfitobacterium* spp. as indi- cated by PCR amplification of *Desulfitobacterium* specific 16S ribosomal RNA gene sequences might be related to the sulfur cycle rather than the halogen cycle, due to its ability of reducing sulfite, thiosulfate and sulfur. In this work, the natural production of organohalides, e. g. in forest soil, was not taken into consideration.

No effort has been made so far to isolate indigenous dehalogenating anaerobes from forest soil. Most of the well characterized dehalogenaing bacteria are isolated from sources probably polluted by anthropogenic activities. *D. hafniense* strain DCB-2 was isolated from municipal sludge (Madsen and Licht, 1992), *Sulfurospirillum multivorans* was from activated sludge (Scholz-Muramatsu *et al.*, 1995), *Desulfitobacterium frappieri* strain TCE1 and *Desulfitobacterium* spp. strain PCE1 were from PCE-contaminated soil (Gerritse *et al.*, 1996; Gerritse *et al.*, 1999).

Here, an anaerobic *O*-demethylating and dechlorinating enrichment culture was obtained from unpolluted forest soil, where lignin-degrading fungi were present. Furthermore, a pure culture of anaerobe with both *O*-demethylation and dehalogenation activities was isolated from this enrichment culture and identified as *D. hafniense* with a high similarity to *D. hafniense* strain DCB-2. Since the laboratory strain *D. hafniense* DCB-2 (originally isolated from municipal sludge) was found to utilize fungal halogenated metabolites as substrates, the isolation of a bacterium closely related to this strain from forest soil reinforces the hypothetic interaction between anaerobic organohalide respirers and lignin-degrading fungi, the role of natural organohalogen in the evolution of dehalogenating microbes, as well as the important role of the dehalogenating anaerobes in the terrestrial halogen cycle (Fig. 4.5).

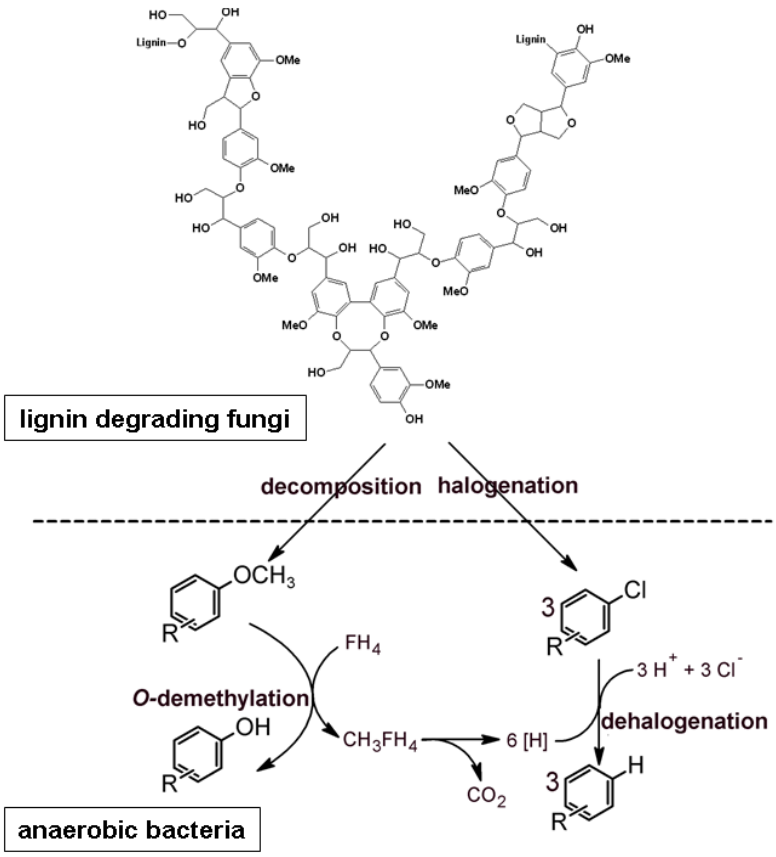

Fig. 4. 5 Scheme of putative microbial halogen cycle in forest soil.

4.4 Outlook

Brominated ethenes were for the first time found to be respiratory substrates for the organohalide respirer *S. multivorans*. The study with the purified PCE dehalogenase from *S. multivorans* and *D. hafniense* strain PCE-S has shown that the dehalogenation of brominated ethenes was proceeded at a lower stereospecificity than that of their chlorinated analogues and that the carbon backbone of the substrate had a more significant effect on the reaction mechanism than the halogen type. Since no stable radical reaction intermediates appeared to be formed during the dehalogenation of halogenated ethenes, a mechanism other than the radical mechanism reported for the dehalogenation of halogenated propenes was assumed and remains to be revealed.

The versatile peroxidases of *B. adusta* strain Ud1 were found to have brominating activity. The chlorinating enzyme responsible for the synthesis of the halogenated metabolites however remains unknown and needs further attempts. Probably it is a substrate-specific enzyme that could not be detected with the haloperoxidase assay using monochlorodimedone as substrate.

Fluoride-dependent substrate oxidation activities were for the first time observed here for manganese peroxidases (MnPs) in the absence of manganese. A hypothesis of fluoride activation of a long-range electron pathway was brought up to explain the fluoride-dependent reactions mediated by MnP. To provide direct evidence for this hypothesis, the amino acid sequence as well as the crystal structure of the MnP-1 from *B. adusta* strain Ud1 should be revealed. The reason for the putative activation of the long-range electron transfer pathway is also not clear. In natural environments such as soil, the fluoride concentration is low (e. g. lower than 0.2 ppm soluble fluoride in agricultural soil) (Larsen and Widdowson, 1971). However, it could not be excluded that the binding of some anions other than fluoride, which are more abundant and more readily available in nature, might activate the long-range electron transfer pathway as fluoride does.

The interaction between aerobic halogenating fungi and anaerobic dehalogenating bacteria has been assumed based on the findings that the anaerobic *D. hafniense* strain DCB-2 was able to *O*-demethylate and dehalogenate the halogenated metabolites of the lignin degrading fungus *B. adusta* strain Ud1 and that the organohalide respirer *D. hafniense* was found in unpolluted forest soil, where lignin degrading fungi were present. To get more direct evidence, a co-culture system was set up (Fig. 4.6).

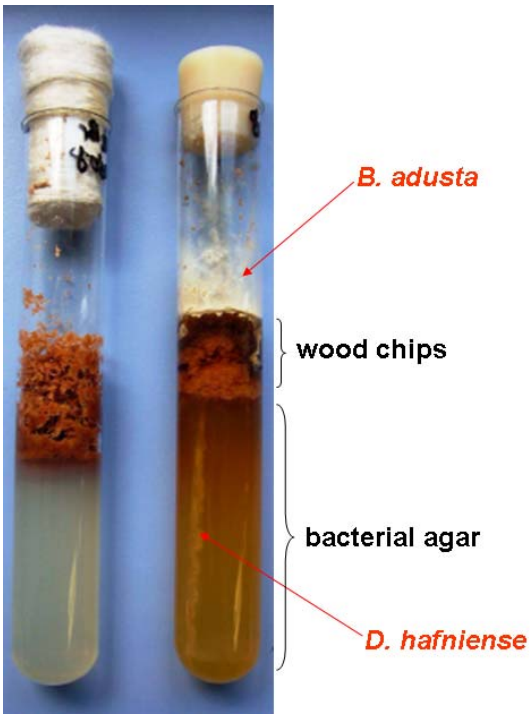

Fig. 4.6 Co-culture setup of aerobic lignin degrading fungi and anaerobic dehalogenating bacteria. Left: control without inoculation of microorganisms; right: after growth of fungi (*B. adusta* strain Ud1) and bacteria (*D. hafniense* strain DCB-2).

The system was composed of a layer of bacterial agar medium with a neutral pH on the bottom and a layer of wood chips moistened with sterile water and inoculated with fungi on the top. An anoxic environment was created in the tube upon the growth of the fungi. The bacteria were then inoculated with a long needle through the fungal layer to the bacterial agar. In this way, the different demand of oxygen for the growth of aerobic fungi and anaerobic bacteria is fulfilled and the growth of both microorganisms was observed in one system (Fig. 4.6). However, the cross-feeding was not studied so far. On one hand, the complicated fungal metabolites as well as the acidic pH fungal culture fluid might have an inhibitory effect on bacterial growth; on the other hand, the concentration of the halogenated compounds produced by fungi might not be sufficiently high to support growth of the bacteria. The development of an improved co-culture experimental setup is currently underway.

5. References

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Acknowledgements

Prof. Dr. Gabriele Diekert introduced me to the field of Anaerobic Microbiology and Enzymology. I thank her sincerely for her nice advice, kind help and perfect supervision of my dissertation. Frequent and intense discussions with her made my work go into depth and helped me to become a mature researcher.

I am very grateful to Dr. Anke Schilhabel and Dr. Jörg Nüske, who have supervised my doctoral research. Anke was not only an excellent advisor in my research work, but also she warmheartedly introduced me to living in Germany. I have learnt a lot from Dr. Nüske about fungal peroxidases and I am grateful for his kind help in proof-reading of this thesis and translating the summary. Dr. Torsten Schubert has also helped me a lot. I am especially thankful for his intensive proof-reading of the Materials and Methods section of this thesis.

I am lucky to have had Markus Kohn, Sandra Studenik, Sandra Kreher, Raffael Rubick, Anika Reinhold, Sandra Hufnagel and Dr. Roland Schmitz as my colleagues in the laboratory. I fondly remember working with them. The discussions with them helped to advance my experiments. I am very grateful to Yvonne Greiser and Peggy Brand for their technical assistance in the laboratory. I would also like to thank our secretary Sabine Schein and the ILRS coordinator Dr. Dorit Schmidt for their assistance in the study program and guidance into life in Jena.

My sincere thanks are given to Prof. Dr. Wilhelm Boland, Dr. Dieter Spiteller, Dr. Stefan Bartram and Kerstin Ploss (Department of Bioorganic Chemistry, MPI for Chemical Ecology) for their help in performing mass spectrometry and NMR analysis and for the valuable discussions. I appreciate the help provided by Dr. Bernhardt Schlott (Department of Biochemistry, FLI) in N-terminal sequencing and Maria Poetsch as well as Dr. Olaf Kniemeyer (Department of Molecular and Applied Microbiology, HKI) in MALDI-TOF analysis of the proteins. I thank Dr. René Ullrich for kindly providing the crude fractions of several fungal peroxidases.

I will never forget all my good friends in Jena. Here, I will not list their names. Without them, my life would lose much color.

Finally, I would like to express my gratitude to my parents and my brother. They always supported and encouraged me to study abroad. There are many thanks and apologies to them in my heart. Without their selfless support and deep love, this dissertation could not have been finished on time. Thank you!

SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, Lidan Ye, geboren am 01.08.1983, das mir die für die Biologisch-Pharmazeutische Fakultät geltende Promotionsordnung bekannt ist. Ich habe die vorliegende Dissertation selbstständig angefertigt und außer den ange- gebenen keine Hilfsmittel, persönliche Mitteilungen oder Quellen eingesetzt. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Es wurden von mir keine geldwerten Leistungen erbracht, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Diese Dissertation wurde nur dem Fakultätsrat der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena und keiner anderen Hochschule zur wissenschaftlichen Prüfung oder zur Dissertation eingereicht. Diese Arbeit ist weder identisch noch teilidentisch mit einer Arbeit, welche der Friedrich-Schiller-Universität Jena oder einer anderen Hochschule zur Dissertation vorgelegt worden ist.

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Publications and poster presentations

1. Lidan Ye, Jörg Nüske, Dieter Spiteller, René Ullrich, Wilhelm Boland, Gabriele Diekert. Fluoride activation - a challenge to Mn dependency of manganese peroxidase. In preparation.

2. Lidan Ye, Anke Schilhabel, Stefan Bartram, Wilhelm Boland, Gabriele Diekert. Reductive dehalogenation of brominated ethenes by *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* PCE-S. Environmental Microbiolgy, in press.

3. Lidan Ye, Anke Schilhabel, Roland Schmitz, Gabriele Diekert. Reductive dehalogenation of dibromoethene by *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* PCE-S. Poster VAAM Conference 2008

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